Vol. XXXVIII March, 1949 No. 3

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

This issue in two Parts

PART I—MARCH JOURNAL, SCIENTIFIC EDITION

Justin L. Powers, Editor

Committee on Publications

George D. Beal, *Chairman*
B. V. Christensen
Robert P. Fischelis
Don E. Francke
Hugo H. Schaefer

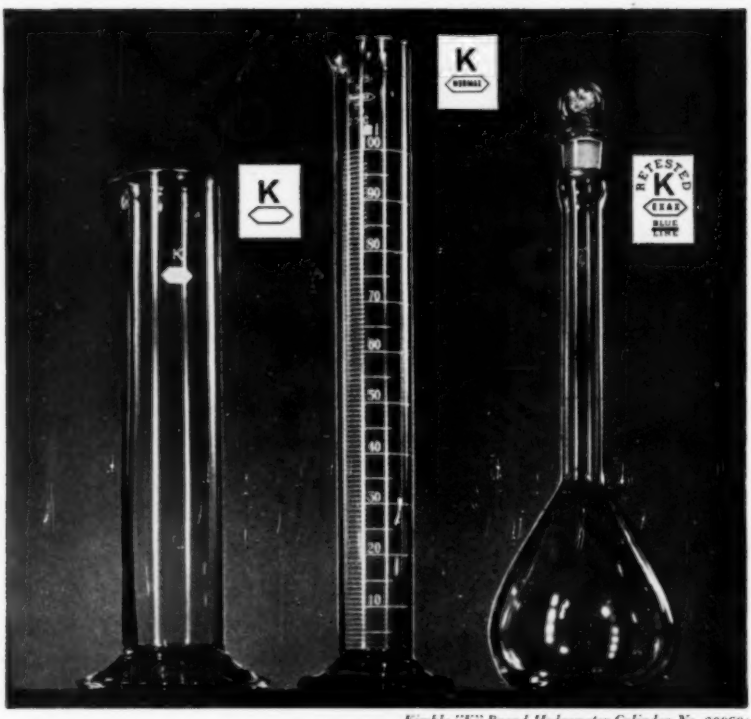
Editorial Advisory Board

L. W. Busse
John E. Christian
T. C. Daniels
L. W. Hazleton
E. J. Hughes
H. W. Youngken, Jr.

CONTENTS OF THIS ISSUE

The Stability of Thiamine Hydrochloride and Mononitrate in Parenteral Vitamin B Complex and Iron Solutions..... 119
Abraham Taub, Irving Katz and *Martin Katz*
Isolation of Oleanolic Acid and Ursolic Acid from *Thymus Vulgaris*, L..... 122
Edward J. Rowe, Jack E. Orr, A. H. Uhl and *Lloyd M. Parks*
A Toxicological Study of *p*-Dichlorobenzene..... 124
Arthur G. Zupko and *Leroy D. Edwards*
Standard Tolerances for Pharmaceutical Compounding. A Basis for Their Establishment. II. Liquid Preparations 131
Samuel W. Goldstein
The Assay of Relatively Pure Benzylpenicillin by Ultraviolet Absorption..... 138
A. A. Colon, G. E. Herpich, J. D. Neuss and *H. A. Frediani*
A Chromatographic Study of Pyrethrum Flowers..... 143
G. H. Hamor, J. F. Suchy and *C. H. Waldon*
Modified Assay Methods for Crude Drugs Involving the Removal of Interfering Substances by Enzymatic Digestion. II. Modified Assay Method for Belladonna Root..... 144
Henry Louis Verhulst, Louis Arrigoni and *Louis Fischer*
The Antibacterial Properties of Cyanine..... 146
Donald W. McKinstry
Experiments on the Constitution of Umbellatine..... 149
R. Chatterjee
The Synthesis of Some Esters of *p*-Carboxybenzamidine..... 154
Frank E. Di Gangi and *Ole Gisvold*
The Spectrophotometric Determination of the Dissociation Constants of Theophylline, Theobromine and Caffeine..... 158
Arthur Turner, Jr. and *Arthur Osol*
Conductometric Studies of Certain Salts of Penicillin..... 161
Frank M. Goyan
Routine "Safety" Tests on Drug Products and the Organization and Maintenance of a Requisite Mouse Colony..... 165
John H. Brewer
The Synthesis of Some Effective Antioxidants and Antiseptics..... 169
Akira Asano and *Ole Gisvold*
Note on the Determination of Estrone, Equilin and Equilenin by Infrared Spectrophotometry..... 173
Frederick C. Nachod, E. T. Hinkel, Jr., and *Calvin Zippin*
Vitamins B_{12}. A Note on the Stability of Thiamine Hydrochloride in Aqueous Solution in the Presence of Iodine and Potassium Iodide..... 174
Hubert W. Murphy and *John M. Goodyear*
Book Reviews..... 175
Advertising..... Cover pages 2, 3, 4, and pp. I–VI

Copyright 1949, by the American Pharmaceutical Association



Kimble "K" Brand Hydrometer Cylinder No. 20060;
Kimble "NORMAX" Precision Graduated Cylinder No. 20026, 100 ml;
Blue Line "EXAX" Retested Flask No. 28015, 200 ml.

LOOK FOR THE KIMBLE "K"
the visible guarantee of invisible quality

• Kimble "K" brand ungraduated glassware has been first choice among laboratory technicians for many years. It is accurately made from mold-blown blanks and machine-drawn tubing . . . retempered for maximum durability.

Kimble "NORMAX" is Science's No. 1 PRECISION graduated glassware . . . calibrated, retested and certified to meet requirements of the National Bureau of Standards. "NORMAX" is the symbol of utmost accuracy.

Kimble Blue Line "EXAX" Retested is the most widely used graduated ware. Its accuracy is assured by expert craftsmanship and *individual retesting*. Tolerances are sufficiently small for most laboratory procedures.

Kimble Glassware can be secured from your laboratory supply dealer

KIMBLE GLASS Toledo 1, Ohio
Division of Owens-Illinois Glass Company

ANNOUNCEMENT REGARDING SUPPLEMENT

In former years the "Proceedings of the American Pharmaceutical Association," the "Yearbook of the American Pharmaceutical Association," the JOURNAL of the A. PH. A. and the Proceedings number of the JOURNAL of the A. PH. A. included the list of members of the A. PH. A., the list of officers of the ASSOCIATION and its Sections since their beginning, the list of meeting places from the time of organization of the ASSOCIATION and the Constitution and By-Laws, Code of Ethics and similar information.

This information has not been published since December 1945. Since it is of interest to all members, it has been segregated into a single volume which is being published as Part II of this issue of the Scientific Edition of the JOURNAL.

The information in this Supplement has historical value and is of importance to officers and members of committees. It has therefore been brought up to date and it is expected that it will be published annually again from now on.

The Supplement includes the official roster and directory of officers and committees for 1948–49 along with current authentic lists of boards of pharmacy and their secretaries, state pharmaceutical associations and their secretaries, the lists and officers of the local and student branches of the ASSOCIATION, and a list of national, sectional, state and local publications in the field of pharmacy.

There is also a list of national pharmaceutical associations and related professional societies, which is referred to frequently by members of the ASSOCIATION in their general and specialized activities.

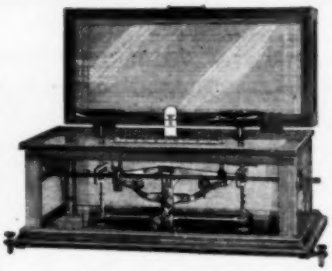
Proceedings of the House of Delegates, the General Sessions and the Sections of the ASSOCIATION will appear separately and will be made available to those who are especially interested in them. The general reports of our conventions in the Practical Pharmacy Edition of the JOURNAL have carried annual reports and addresses of officers and reference to committee actions and resolutions, but from an historical point of view it has been considered desirable to collect the "proceedings" of the meetings in a single volume. This has not been the continuous policy of the ASSOCIATION since its JOURNAL was established. For a time, the monthly issues of the JOURNAL carried certain portions of the proceedings. Later on, the proceedings were concentrated in one volume. It is expected that the 1946–47 proceedings will appear in a single volume, but that the 1948 proceedings will be made available in sections and will be sent in that form to those concerned and to members who express an interest in receiving these publications.

It should be understood that Part II of this issue of the JOURNAL is not a volume of "proceedings," but is specifically a list of members and a record of the more permanent information desired from time to time by members for various purposes.

Robert P. Fischelis, *Secretary*
AMERICAN PHARMACEUTICAL ASSOCIATION

No. 540 Scale shown illustrated has
Capacity—4 oz. (120 grams)
Sensitivity—1/10 grain
Finish—Genuine light mahogany with chrome plated parts
Price—$130.00 (subject to change without notice)

PROMPT DELIVERY
on this as well as other class A scales
No. W35 (all glass in lid)..... $90.00
No. W 33 .....75.00



High quality Prescription Scale made by
HENRY TROEMNER
Manufacturer of the Finest Scales for the Druggist Since 1840
911 Arch Street
Philadelphia 7, Pa.
(*Send for Circular*)

DETROIT COLLEGE OF PHARMACY AND CHEMISTRY
A co-educational institution offering degree courses in Pharmacy (B.S. Pharm.) and Chemistry (B.S. Chem.). Graduation from an accredited High School or the equivalent is required for admission.
Applicants not candidates for a degree may enroll as special students for courses in BIOLOGY, CLINICAL LABORATORY METHODS, BACTERIOLOGY, and others, provided evidence of prerequisite credit is presented. Each year through the co-operation of the College, the alumni, and Detroit pharmacists, many students are placed in part-time positions. Write for catalog.
FULLY ACCREDITED REHABILITATION TRAINING FOR RETURNED VETERANS
DETROIT INSTITUTE OF TECHNOLOGY
2020 Witherell Detroit 26 Michigan

The Massachusetts College of Pharmacy
Longwood Avenue, Boston

Graduate study in Boston has always appealed strongly to students in the field of medicine and public health. The environment is stimulating and attracts students from all parts of the world. Library, laboratory, hospital, and other facilities for graduate work are outstanding in this area.

The Massachusetts College of Pharmacy is located in a great medical and educational center It offers unusual opportunities to graduates of colleges of pharmacy who are interested in further study in pharmacy, pharmaceutical chemistry, and pharmacognosy. A few fellowships are available.

For additional information, write to HOWARD C. NEWTON, Dean.

***Philadelphia* COLLEGE OF PHARMACY AND SCIENCE**
43rd St. and Kingsessing Ave., Philadelphia, Penna. Founded in 1821
Courses of study offered leading to B.Sc. degrees in Pharmacy, Chemistry, Bacteriology and Biology. Graduate studies leading to M.Sc. and D.Sc. Coeducational.

Published by the American Pharmaceutical Association under supervision of the Committee on Publications with the approval of the Council. Publication office, 20th and Northampton Sts., Easton, Pa. Editorial office: Scientific Edition, 2215 Constitution Ave., Washington 7, D. C.

Annual Subscription—Journal of the American Pharmaceutical Association, complete (both editions): United States and Pan America, $7.00; Canada, $7.70; other foreign, $8.00; members of the American Pharmaceutical Association with dues, $4.00. Each edition, Scientific Edition or Practical Pharmacy Edition: United States and Pan America, $4.00; Canada, $4.35; other foreign, $4.50. Single numbers, either edition: United States and Pan America, $0.35; Canada, $0.40; other foreign, $0.50.

Claims for missing numbers will not be allowed (1) if received more than 60 days from date of issue, (2) if loss was due to failure to give 30 days notice of change of address, or (3) if reason for claim is "missing from files".

Entered as second-class matter January 23, 1917, at the Post Office at Easton, Pa., under the act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the fifth; Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

Robert P. Fischelis, *Secretary and General Manager*

Compoundable Chemicals for Precision Compounding

Ichthymall

(Ichthammol N.F. VIII)

ICHTHYMALL® MALLINCKRODT—*No interruption in supply to pharmacists for over 20 years.*

ICHTHYMALL MALLINCKRODT is the purified ammonium salt of ichthysulfonic acid, prepared by the sulfonation of the oil obtained by the distillation of bituminous shale—rich in the oily residue left after fish have become fossilized.

This purified ammonium ichthysulfonate is a thick viscid liquid, reddish brown to brownish black in color. *It is distinguished by its high, uniform content of organically combined sulfur.*

Ichthymall conforms to all N.F. VIII specifications.

SOLUBLE IN: *water, glycerine, mixtures of alcohol, water and ether.*

PARTIALLY SOLUBLE IN: *alcohol, ether.*

MISCIBLE IN ALL PROPORTIONS WITH: *oils, ointment bases, petrolatum, lanolin, lard, etc.*

ICHTHYMALL is incompatible with potassium iodide, resorcinol, mercuric chloride, alkali hydroxides and carbonates, and acids.

Order ICHTHYMALL MALLINCKRODT from your wholesaler—TODAY.

1-oz., ¼-lb., 1-lb. and 5-lb. bottles. ®T. M. Reg. U. S. Pat. Off.

82 Years of Service to Chemical Users

Mallinckrodt Chemical Works

UNIFORM, DEPENDABLE PURITY

Mallinckrodt St., St. Louis 7, Mo.
72 Gold St., New York 8, N. Y.
CHICAGO • CINCINNATI • CLEVELAND
LOS ANGELES • MONTREAL
PHILADELPHIA • SAN FRANCISCO

MALLINCKRODT CHEMICAL WORKS, DEPT. JAP.
SECOND & MALLINCKRODT STREETS, ST. LOUIS 7, MISSOURI
72 GOLD ST., NEW YORK 8, N. Y.

For distribution to physicians on my mailing list, please send me_____copies of Mallinckrodt ICHTHYMALL *booklet listing typical prescriptions used by many physicians.*

PHARMACY'S NAME_____

PHARMACY'S ADDRESS_____

CITY_____

WHOLESALER'S NAME_____

LABCO PRODUCTS
FOR VITAMIN RESEARCH

LABCO VITAMIN-FREE CASEIN:—A highly purified casein, widely used in the preparation of rations for vitamin assay and research purposes.

LABCO LACTALBUMIN:—A high quality animal protein for use where freedom from vitamin entities is not a prerequisite. An edible protein suitable for use in the human dietary.

SEND FOR FURTHER INFORMATION. For other Labco products, their use and analysis address Dept. A

THE BORDEN COMPANY
Labco Products Department
350 Madison Avenue, New York 17, N. Y.

APPLICATION FOR ACTIVE MEMBERSHIP IN THE AMERICAN PHARMACEUTICAL ASSOCIATION

Approving of its objectives, I hereby apply for active membership in the AMERICAN PHARMACEUTICAL ASSOCIATION. I enclose $10 to cover annual dues for 12 months beginning with the month following my election to membership. Membership includes subscription to the JOURNAL OF THE A. PH. A. which is published monthly in two editions, "*Practical Pharmacy*" and "*Scientific.*" Subscription to the JOURNAL for non-members is $4 for each Edition or $7 combined. Members of the A. PH. A. receive a 50% discount on the regular subscription rate of each Edition. Therefore $2 for each edition (total $4) is allocated for subscription to the JOURNAL.

Name in Full (Miss—Mr.—Mrs.—) _____

Street and No. _____

City _____ Zone _____ State _____

Date of Birth _____ State Assn. member in _____

Graduate of _____ Year _____ Degree _____

Licensed Pharmacist in (states) _____

Please indicate by check mark below your classification(s) according to present employment:

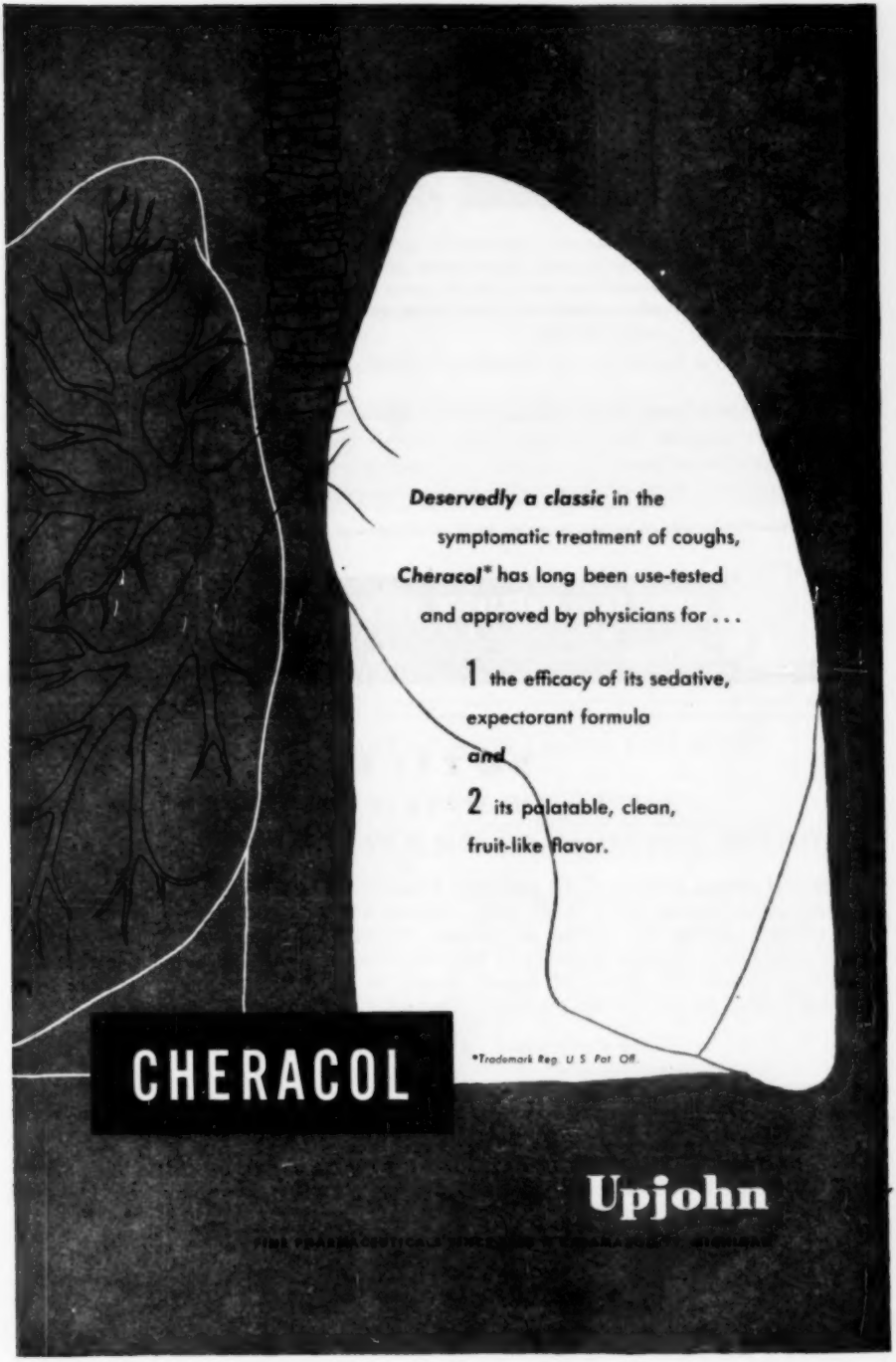
_ Retail Pharmacist; _ Hospital Pharmacist; _ Government Pharmacist; _ Teacher; _ Wholesaler; _ Manufacturer; _ Representative; _ Research or Control Chemist; _ Association Official; _ Student; _ Other _____

Signature of Applicant _____

This application is endorsed by the following members of the AMERICAN PHARMACEUTICAL ASSOCIATION:

Name _____ Name _____

Please send this application with check or money order for $10 to:
ROBERT P. FISCHELIS, Secretary, American Pharmaceutical Association
2215 Constitution Avenue N. W., Washington 7, D. C.


Deservedly a classic in the
symptomatic treatment of coughs,
Cheracol® has long been use-tested
and approved by physicians for . . .
1 the efficacy of its sedative,
expectorant formula
and
2 its palatable, clean,
fruit-like flavor.
CHERACOL
*Trademark Reg. U. S. Pat. Off.
Upjohn

CHOLESTEROL IN ITS MOST ACTIVE AND EFFICIENT FORM

Thousands of Emulsifiers but . . .

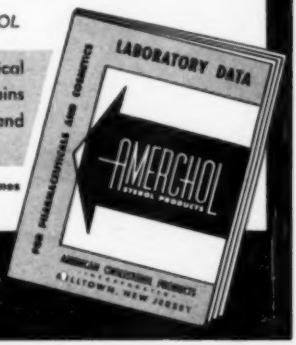
the AMERCHOLS ARE NATURALLY DIFFERENT!

These non-ionic, surface-active agents are NATURALLY good for the skin. Of NATURAL origin, they represent odorless, color-free forms of cholesterol and related rare sterols which we isolate from animal tissues. The Amerchols are efficient, highly active EMULSIFIERS, PENETRANTS and EMOLLIENTS for DRUGS and COSMETICS.

WE KNOW OF NO CASE OF ALLERGY DUE TO AN AMERCHOL

For complete information write for your copy of our new technical booklet, "Laboratory Data" on your company letterhead. Contains technical data and formulas for the use of the Amerchols in o/w and w/o emulsions, ointments, lotions, creams, hair preparations, etc.

Our research laboratories are available to you for advice and information at all times



AMERICAN CHOLESTEROL PRODUCTS
• INCORPORATED •
MILLTOWN — NEW JERSEY

NOTICE

PAPERS FOR SECTION MEETINGS OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

The next annual meeting of the American Pharmaceutical Association will be held in Jacksonville, Florida, April 24–30, 1949. Members planning to present papers before any Section during this meeting are earnestly requested and strongly urged to notify the appropriate Section secretary of their intention promptly. Titles and abstracts (not to exceed 250 words) of papers should be submitted promptly in order to insure a place on any Section program. Send titles and abstracts of papers to:

SECTION SECRETARIES

SCIENTIFIC
RAY S. KELLEY, Secretary
179 Longwood Avenue
Boston 15, Mass.

PHARMACEUTICAL ECONOMICS
E. J. IRELAND, Secretary
6363 St. Charles Avenue
New Orleans 15, La.

EDUCATION AND LEGISLATION
RALPH W. CLARK, Secretary
University of Kansas
School of Pharmacy
Lawrence, Kan.

PRACTICAL PHARMACY
ELMER M. PLEIN, Secretary
University of Washington
College of Pharmacy
Seattle 5, Wash.

HISTORICAL PHARMACY
H. GEORGE WOLFE,
35-16—76th Street
Jackson Heights,
L. I., N. Y.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXVIII | *MARCH, 1949* | NUMBER 3 | CONSECUTIVE NO. 6

The Stability of Thiamine Hydrochloride and Mononitrate in Parenteral Vitamin B Complex and Iron Solutions*

By ABRAHAM TAUB,† IRVING KATZ,‡ and MARTIN KATZ‡

Vitamin B_1, in parenteral solutions in admixture with riboflavin and niacinamide, exhibits maximum stability with respect to potency and clarity of solution at *p*H 4 under a nitrogen atmosphere. In admixture with iron compounds, vitamin B_1 is more stable in the presence of ferrous gluconate than of iron peptonate or ferric ammonium citrate. The optimum *p*H is 4. Of the specific grades studied, thiamine mononitrate is somewhat more stable than the hydrochloride in the above solutions.

ALTHOUGH thiamine hydrochloride in aqueous solution has been reported as stable below *p*H 5.5 (1), and capable of being sterilized at 120° for twenty minutes at *p*H 3.5 (2), evidence of minute decomposition in the form of discoloration and slight sedimentation, significant in parenteral solution manufacture, often becomes apparent within a few weeks to several months after preparation of Injection Thiamine Hydrochloride.

The exact mechanism of this decomposition, which is not correlated with any significant drop in potency, is not clearly understood. Oxidation leading to thiochrome formation seems to play only a subordinate role. Cleavage of the thiazole ring may give rise to the formation of colloidal solutions of sulfur and other degradation products, which under the influence of higher temperature during ampul sealing, sterilization or storage, or of catalysts in the form of trace metallic impurities in reagents employed, are coagulated to form visible particles or sediment. It has been estimated that decomposition of as little as 0.0001 per cent of thiamine can result in a noticeable precipitate (3).

The source of thiamine hydrochloride is an important factor in the preparation of a stable solution. Conformity to U. S. P. specifications is not in itself an indication of satisfactory quality for parenteral use. This has been recognized in a collaborative study in which there has been

* Received July 8, 1948, from the Department of Pharmaceutical Chemistry, Columbia University College of Pharmacy, New York City.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
† Professor of Pharmaceutical Chemistry, Columbia University College of Pharmacy.
‡ American Foundation for Pharmaceutical Education Research Fellow.

recommended a special ampul grade of thiamine (4). Such grades represent specially recrystallized batches of thiamine hydrochloride, empirically evaluated on the basis of satisfactory quality after several weeks' storage in solution. Although such grades have been commercially available, they are not the final answer to the problem. Ampul grades, though satisfactory when first obtained, frequently deteriorate slightly upon storage in the dry state after several months, even when kept below 20° under carbon dioxide. Ampul grades from the same sources of supply have been known to fluctuate markedly in quality indicating that the exact nature of the mechanism of induction of decomposition is not clearly understood.

The decomposition of thiamine injection is further aggravated when other B vitamins are added (5), or when certain iron compounds are introduced into the solution. Few published studies of these combinations appear, though they have been the subject of considerable empiric research as production problems in industry. That these studies have not met with marked success is indicated by the introduction of lyophilized B vitamin parenteral products to obviate the factor of deterioration due to the aqueous vehicle. Costliness of equipment and other factors have precluded the widespread adoption of this process.

EXPERIMENTAL

The introduction of thiamine mononitrate for experimental use in 1947, and preliminary studies pointing toward its greater stability than the hydrochloride (6), suggested the present study of comparative stability of the two salts in some commonly used vitamin B complex and iron solutions for parenteral use.

A comparison of some properties of both compounds is presented in Table I.

The acute oral and intravenous toxicity in mice and in rats is the same for both compounds (6, 7).

In solution at *p*H 4 the potency of the thiamine mononitrate has been reported as unchanged after more than twelve months; solutions in ampuls remain clear, with only slight discoloration, twenty-one months after autoclaving, if stored in the dark at room temperature (6).

In the present study the objectives were to determine the effect on stability of thiamine hydrochloride and nitrate solutions produced by (*a*) the presence of riboflavin and niacinamide at different *p*H levels and under an atmosphere of air or nitrogen; and (*b*) the presence of iron compounds, namely, the gluconate, peptonate, and green iron and ammonium citrate, at different *p*H levels.

A concentration of 50 mg. thiamine salt per cc. was used in all samples. The *p*H range was limited to 3–5. A lower *p*H gives rise to pain on injection; at higher *p*H, deterioration is rapid. In the B complex solutions, 2 mg. riboflavin per cc. was dissolved, using 200 mg. niacinamide per cc. to solubilize the riboflavin (8). Riboflavin and niacinamide were refluxed in solution for several minutes at boiling temperature, cooled to 25°, the thiamine salt added and the *p*H adjusted with hydrochloric acid or sodium hydroxide solution. Solutions after adjustment to desired volume were filtered aseptically through Selas 03 porcelain filter candles and filled into thoroughly washed clear glass Kimble ampuls, which had been dried in a dust-free atmosphere and sterilized. One-half of the lot was sealed under an atmosphere of purified nitrogen. Special care was exercised during filling and sealing to avoid formation of charred necks. Solutions in ampuls were sterilized at 100° for one hour. Although in commercial practice solutions containing thiamine are commonly sterilized by bacteriological filtration, in the present study the higher temperature was employed so that differences in stability between the thiamine nitrate and hydrochloride could be noted more speedily.

In the preparation of the thiamine and iron solutions the following variations from the above procedure were employed. Each solution contained in addition to 50 mg. of thiamine salt, 130 mg. of one of the iron compounds selected and 10 mg. of sodium citrate per cc., the latter to help keep the iron compound in solution. Solutions were prepared without heat. All ampuls were sealed under air and were sterilized at 15 lbs. for twenty minutes.

The following grades of chemicals were used: Thiamine Mononitrate, "for experimental use" (Merck); Thiamine Hydrochloride, ampul grade AA (Pfizer); Riboflavin U. S. P.; Niacinamide U. S. P.; Green Ferric Ammonium Citrate N. F.; Iron Peptonate N. F.; Ferrous Gluconate N. F.

All solutions in ampuls were tested for sterility assayed by the thiochrome method, and inspected for clarity. Those free from suspended matter were stored in the dark, either at 45° for three weeks, or at room temperature (22° ± 5°) for one year.

The results are indicated in Tables II and III.

Although it was not the purpose of this study to determine the stability of the riboflavin in the above

TABLE I.—COMPARISON OF THIAMINE HYDROCHLORIDE AND NITRATE

	Thiamine Hydrochloride	Thiamine Nitrate
Formula	$C_{12}H_{17}ON_4SCl \cdot HCl$	$C_{12}H_{17}ON_4S \cdot NO_3$
Molecular weight	337.27	327.36
Potency	1 mg. = 333 I. U.	1 mg. = 343 I. U.
Description	White, crystalline powder with a slight odor	White, crystalline powder, nearly odorless
Moisture content (approx.)	4%	0.1%
Solubility in water	1 Gm./cc.	0.027 Gm./cc.[a]
Reaction of saturated solution	*p*H 2.5–3.0	*p*H 6.8–7.0

[a] At *p*H 4 the solubility is increased to 0.18 Gm./cc.

TABLE II.—RELATIVE STABILITY OF PARENTERAL SOLUTIONS CONTAINING THIAMINE HYDROCHLORIDE OR MONONITRATE IN THE PRESENCE OF RIBOFLAVIN AND NIACINAMIDE

pH	Thiamine Salt	Atmosphere in Ampul	Three Weeks at 45° % Thiamine Decomposition	Three Weeks at 45° Sediment[a]	One Year at 22° ± 5° % Thiamine Decomposition	One Year at 22° ± 5° Sediment[a]
3.0	Chloride	Air	25	4	5	4
3.0	Chloride	Nitrogen	24	4	5	3
3.0	Nitrate	Air	16	4	3	4
3.0	Nitrate	Nitrogen	14	4	2	2
4.0	Chloride	Air	23	3	8	3
4.0	Chloride	Nitrogen	23	3	6	3
4.0	Nitrate	Air	16	2	6	2
4.0	Nitrate	Nitrogen	15	2	4	1
5.0	Chloride	Air	30	2	21	2
5.0	Chloride	Nitrogen	27	2	15	2
5.0	Nitrate	Air	21	1	18	1
5.0	Nitrate	Nitrogen	17	1	12	0

[a] Degree of sedimentation is expressed numerically as follows: 0, clear; 1, very faint spiral cloud formed upon rotation of upright ampul; 2, slight cloud; 3, light sediment; 4, heavy sediment.

TABLE III.—RELATIVE STABILITY OF PARENTERAL SOLUTIONS CONTAINING THIAMINE HYDROCHLORIDE OR MONONITRATE IN THE PRESENCE OF VARIOUS IRON COMPOUNDS

Iron Compound	Thiamine Salt	pH	Three Weeks at 45° % Thiamine Decomposition	Three Weeks at 45° Sediment[a]	One Year at 22° ± 5° % Thiamine Decomposition	One Year at 22° ± 5° Sediment[a]
Iron peptonate	Chloride	3.0	9	4	2	3
	Chloride	4.0	11	3	3	2
	Chloride	5.0	23	2	9	2
	Nitrate	3.0	9	2	2	2
	Nitrate	4.0	10	2	3	2
	Nitrate	5.0	14	1	10	1
Ferrous gluconate	Chloride	3.0	9	4	4	4
	Chloride	4.0	11	3	6	2
	Chloride	5.0	13	2	10	2
	Nitrate	3.0	11	3	4	3
	Nitrate	4.0	10	2	5	0
	Nitrate	5.0	13	2	10	0
Ferric ammonium citrate	Chloride	3.0	11	4	6	4
	Chloride	4.0	21	4	15	4
	Chloride	5.0	23	4	16	4
	Nitrate	3.0	10	4	4	4
	Nitrate	4.0	14	4	6	4
	Nitrate	5.0	15	4	9	4

[a] See footnote, Table II.

solutions, it is of interest to note that in a period of a year at room temperature the decomposition of riboflavin was found to be about 7% under air and 4% under nitrogen at pH 3, 9% under air and 7% under nitrogen at pH 4, and 24% under air and 18% under nitrogen at pH 5. No significant difference was noted between riboflavin decomposition in the presence of thiamine hydrochloride or mononitrate.

In addition to the degree of sediment indicated, a slight filming of the inner glass surface was noted in the iron peptonate and ferric ammonium citrate ampuls. The ferrous gluconate ampuls showed no filming at pH 4 and pH 5.

DISCUSSION AND SUMMARY

Thiamine decomposition in solution, after storage at 45° for three weeks, is more severe than after one year's storage at room temperature. Although the three weeks' test at 45° has been suggested as indicative of a year of shelf life for vitamin tablets and capsules, it is too rigorous an accelerated test for aqueous thiamine solutions.

The thiamine salts studied appear most stable, in the presence of riboflavin and niacinamide, at pH 3 with respect to potency, and are nearly as stable at pH 4. A pH of 5 is not a suitable environment. With respect to sedimentation the reverse is true; best results are obtained at pH 5 and nearly as good results at pH 4. An atmosphere of nitrogen yields slightly more stable solutions. The grade of thiamine mononitrate studied appears slightly superior to the grade of thiamine hydrochloride with respect to potency and clarity. It should be mentioned that preliminary tests of the grade of thiamine hydrochloride selected for this study showed the least amount of sedimentation upon prolonged storage

in aqueous solutions of all available grades at the time of the investigation.

Although optimum results are obtained by use of thiamine mononitrate under nitrogen at *p*H 4, complete clarity was not obtained after a year's storage at room temperature. It should be emphasized, however, that the parenteral solutions in this study were purposely sterilized at 100° or autoclaved to accelerate decomposition. It is reasonable to expect that if heat is avoided and solutions are prepared aseptically by sterile filtration, clarity over a longer storage period will be evident.

With respect to the iron compounds studied, thiamine salts show the poorest results in the presence of ferric ammonium citrate; marked sedimentation occurs under all conditions specified.

In the presence of either ferrous gluconate or iron peptonate, thiamine mononitrate shows little advantage over the hydrochloride with respect to potency or storage; both salts are more stable at *p*H 3–4 than at *p*H 5. However, the mononitrate shows lesser sedimentation than the hydrochloride. Both thiamine salts show less sedimentation at *p*H 5 than at *p*H 3. In the presence of ferrous gluconate, thiamine mononitrate at *p*H 4–5 yields solutions that remain clear after a year's storage at room temperature. At *p*H 4 the mononitrate in the presence of ferrous gluconate shows optimum stability both with respect to potency and clarity.

REFERENCES

(1) Vitamin Reviews: Thiamine Hydrochloride, Merck & Co., Rahway, N. J., 1947, p. 3.
(2) Rosenberg, H. K., "Chemistry and Physiology of the Vitamins," Interscience Publishers, Inc., New York, 1942, p.104.
(3) Vitamin B_1 Parenteral Solutions, Hoffmann-La Roche Inc., Nutley, N. J., 1948, p. 2.
(4) *Proc. Am. Pharm. Mfrs. Assoc.*, pp. 29–30(1945).
(5) *Proc. Am. Pharm. Mfrs. Assoc.*, Scientific Conferences, pp. 133–5(Feb. 1948).
(6) Thiamine Mononitrate, Merck & Co., Rahway, N. J., 1947.
(7) Haley, T. J., *Proc. Soc. Exptl. Biol., Med.*, **68**, 153 (1948).
(8) Frost, D. V., *J. Am. Chem. Soc.*, **69**, 1064(1947).

Isolation of Oleanolic Acid and Ursolic Acid from *Thymus Vulgaris*, L.*

By EDWARD J. ROWE,† JACK E. ORR,‡ A. H. UHL, and LLOYD M. PARKS

***Thymus vulgaris* L. has been investigated for its nonvolatile constituents. Two previously unreported, isomeric triterpene hydroxy-acids were isolated. Oleanolic acid, $C_{30}H_{48}O_3$, was obtained from the petroleum ether extract and characterized through its acetate. Ursolic acid, $C_{30}H_{48}O_3$, was isolated from the ethyl ether extract and identified through its acetate, acetyl-acid chloride, and methyl ester.**

THE CHEMISTRY of *Thymus vulgaris* Linné has, in the past, been largely restricted to the study of its volatile oil and constituents isolated therefrom. Comparatively little has been known of the nonvolatile constituents of the plant aside from the reported presence of salts, gum, resin (1), tannin (2), pentosan (3), and saponin (4). Therefore, further chemical investigation of the nonvolatile portion of this official drug seemed worth while.

In the course of this work, two previously unreported substances were isolated, one from the petroleum ether extract and the other from the ethyl ether extract. The physical appearance, solubility behavior, and high melting points of these substances suggested that they might be members of the polycyclic triterpenoid class of compounds. Proceeding on this assumption, the purified compound from the petroleum ether extract was identified through its acetate as oleanolic acid ($C_{30}H_{48}O_3$), a triterpene hydroxy-acid found in clove buds (caryophyllin), olive leaves, and many other plants. The purified compound from the ethyl ether extract was characterized through its acetate, acetyl-acid chloride, and methyl ester as ursolic acid ($C_{30}H_{48}O_3$), a triterpene hydroxy-acid isomeric with oleanolic acid and found commonly in *uva ursi*.

The only other plant in which the coexistence of these isomers has been reported is the grape

* Received July 22, 1948, from the School of Pharmacy, University of Wisconsin, Madison, Wis.
† Present address: Butler University College of Pharmacy, Indianapolis, Ind.
‡ Present address: University of Utah College of Pharmacy, Salt Lake City, Utah.

(*Vitis vinifera* L.). Markley, Sando, and Hendricks (5) found oleanolic acid in the cuticle of the fruit, while Kuwada and Matsukawa (6) isolated ursolic acid from the cuticle of the leaves of the grape.

EXPERIMENTAL[1]

Isolation of Oleanolic Acid.—Eleven kilograms of finely ground, air-dried drug was extracted by continuous percolation with petroleum ether (Skellysolve "B") for about fifty hours, at which time a greenish substance began to settle out. The percolate was replaced with fresh solvent and extraction continued to exhaustion. The insoluble material, representing about 13% of the total petroleum ether extract, was dark green and readily pulverizable. Crystallization of the crude, green powder from alcohol yielded white, feathery, needle-like crystals. Several recrystallizations from alcohol produced a constant melting point at 307–308°. $[\alpha]_D^{24.5} = +78.85$ (20.8 mg. in 2 ml. $CHCl_3$). When mixed with a known sample of oleanolic acid (m. p. 307–308°), obtained from cloves by the method of Dodge (7), no depression in melting point was observed.

Dodge (7), Wedekind, *et al.* (8), and Markley, *et al.* (5), report a melting point of 310° while Winterstein (9) reports 306–308° for oleanolic acid. Van der Haar (10) reports $[\alpha]_D + 79.70$ ($CHCl_3$), and Wedekind (8) $[\alpha]_D + 79.6$ ($CHCl_3$) for oleanolic acid.

Acetate.—The acetyl derivative was prepared by gently refluxing 0.2 Gm. of the above with a mixture of 10 ml. of acetic anhydride and 0.5 Gm. of fused sodium acetate. The product, after crystallization from alcohol, melted at 262–263°. Mixture with a known sample of acetyl oleanolic acid (m. p. 262–264°) showed no depression of melting point. Dodge (7) reports a melting point of 260–265° and Markley, *et al.* (5) report 264.5–265.5° for the acetyl derivative.

Isolation of Ursolic Acid.—Following the petroleum ether extraction, the marc was exhaustively extracted with ethyl ether. A portion of the light green, readily pulverizable material obtained upon removal of the ether was dried, dissolved in alcohol, decolorized by heating under reflux for ten minutes with Norite, filtered, and cooled to room temperature, whereupon white, needle crystals melting at 278–281° were obtained. Repeated crystallization from ethanol and methanol failed to produce any significant change in melting point. The entire ether extract was thus treated and about 80 Gm. of material of the above melting point was obtained.

The crystals were insoluble in water, 5% HCl, 5% NaOH, and moderately soluble in ether, ethanol, methanol, and chloroform at room temperature. The material dissolved in concentrated sulfuric acid producing a yellow solution. A potassium salt was prepared and found to be insoluble in ether and water, but soluble in alcohol.

Acetate.—The acetyl derivative was prepared by refluxing 10 Gm. of the above for eight and a half hours with a mixture of 50 ml. of acetic anhydride and 100 ml. of glacial acetic acid. The reaction product was crystallized from ethanol and recrystallized four times from methanol and once from acetone, yielding a final, constant melting point of 288–290°. A mixture with a known sample of acetyl ursolic acid (m. p. 290–291.5°) showed no depression of melting point. Sando (11) lists the melting point of acetyl ursolic acid as 289–290°.

Saponification of the Acetate.—The acetate was saponified by refluxing with methyl alcoholic potassium hydroxide for three hours. The reaction product was crystallized from methanol to a constant melting point of 282.5–284°. A mixture with a known sample of ursolic acid (m. p. 282–284°) showed no depression of melting point. A mixed melting point of the saponification product and the acetate showed a depression of 30–35°. Sando (11) and Jacobs (12) report a melting point of 284–285° for ursolic acid.

The optical rotation was determined for both the saponification product and a known sample of ursolic acid with these results: Saponification product $[\alpha]_D^{24.5} = +62.1$ (45.7 mg. in 2 ml. of 0.09 *N* methyl alcoholic KOH, α_D +1.42°, *l* 1 dm.). Ursolic acid—$[\alpha]_D^{24.5} = +62.6$ (40.6 mg. in 2 ml. of 0.09 *N* methyl alcoholic KOH, α_D + 1.27°, *l* 1 dm.).

Acetyl-Acid Chloride.—Two-tenths of a gram of the acetyl derivative was dissolved in 1 ml. of thionyl chloride, allowed to stand for two hours at room temperature, and then refluxed on a steam bath for half an hour. The excess thionyl chloride was distilled off under reduced pressure and the residue crystallized from a mixture of benzene and petroleum ether. Recrystallization from heptane and then from Skellysolve "B" gave white, needle crystals melting at 222°. A mixed melting point with a known sample of acetyl ursolyl chloride (m. p. 223°) showed no depression. Goodson (13) reports a melting point of 224–226° for this compound.

Methyl Ester.—The acid chloride was refluxed for six hours with methanol. The reaction mixture was then poured into water, the precipitate extracted with ether, and solvent removed by distillation. Crystallization of the product from methanol gave crystals melting first between 110–120°, then resolidifying, and finally melting sharply at 169°. After further crystallization only the 169° melting point was observed. Since this is the melting point of methyl ursolate, it was assumed that the acetyl group had been split off. This assumption was confirmed by a mixed melting point with known methyl ursolate (m. p. 170°) which showed no depression, and by refluxing known acetyl ursolyl chloride with methanol. The latter treatment yielded a product melting at 170° and which did not depress the melting point of the unknown.

SUMMARY

1. *Thymus vulgaris* Linné has been investigated regarding its nonvolatile constituents.

2. The substance isolated from the petroleum ether extract has been identified as oleanolic acid, a triterpene hydroxy-acid with molecular formula $C_{30}H_{48}O_3$.

3. The substance isolated from the ethyl ether extract has been identified as ursolic acid, a triterpene hydroxy-acid with molecular formula $C_{30}H_{48}O_3$ and isomeric with oleanolic acid.

[1] All melting points are corrected.

REFERENCES

(1) Hager, H., "Handbuch der Pharmazeutischen Praxis," Vol. 1, 1888, p. 1140.
(2) Moritz, O., "Einführung in die Allgemeine Pharmakognosie," 1936, p. 159.
(3) Hanus, J., and Bien, F., *Z. Untersuch. Lebensm.*, **12**, 395(1906).
(4) Kroeber, L., *Pharm. Monatsh.*, **2**, 72(1924).
(5) Markley, K., Sando, C., and Hendricks, S., *J. Biol. Chem.*, **123**, 641(1938).
(6) Kuwada, S., and Matsukawa, T., *J. Pharm. Soc. Japan*, **53**, 368(1933).
(7) Dodge, F. D., *J. Am. Chem. Soc.*, **40**, 1917(1918).
(8) Wedekind, E., and Schicke, W., *Z. physiol. Chem.*, **195**, 132(1931).
(9) Winterstein, A., and Hämmerle, W., *ibid.*, **199**, 56 (1931).
(10) van der Haar, A. W., *Rec. trav. chim.*, **46**, 796(1927).
(11) Sando, C. E., *J. Biol. Chem.*, **90**, 477(1931).
(12) Jacobs, W. A., and Fleck, E. E., *ibid.*, **92**, 487(1931).
(13) Goodson, J. A., *J. Chem. Soc.*, **1938**, 999.

A Toxicological Study of *p*-Dichlorobenzene*

By ARTHUR G. ZUPKO† and LEROY D. EDWARDS‡,§

A determination of the LD_{50} dose of *p*-dichlorobenzene by the method of Bliss is presented. The results of a hematological study on three species of warm-blooded animals after a series of exposures to *p*-dichlorobenzene vapors indicate that this compound has a selective action on the granulocytes of the blood. Contrary to reports in the literature a histological study of twelve rabbits, thirteen rats, and eight guinea pigs, representing those animals dying or sacrificed after a series of exposures to *p*-dichlorobenzene vapors, revealed comparatively little liver damage or evidence of a definite hepatitis. Every animal, without exception, showed marked and extensive damage to the kidneys.

PARA-DICHLOROBENZENE or 1,4-dichlorobenzene I. U. C.[1] is used extensively in the United States for insecticidal purposes and as a deodorant. In spite of its inception as a fumigant in 1914 (1), there is little information concerning its toxicity in the literature. Recently, however, PDB crystals were compared with DDT spray against fly larvae in outside pits, and proved to be more effective than the more highly publicized latter compound (2). The purpose of this paper is to contribute added toxicological information concerning this increasingly important compound.

The inhalation of PDB vapors in sufficient quantities over a long period of time may produce severe toxic effects in humans. Although the literature is comparatively barren of such reports, Perrin (3) reported the case of a 62-year-old man wearing clothes impregnated with PDB who developed asthenia and attacks of vertigo and whose blood picture showed anemia and hypogranulocytosis with normalization delayed for a long time. The picture was similar to that caused by benzene. Berliner (4) believed that in human subjects the toxic effects of PDB inhalation manifest themselves as hepatitis, loss of weight, and, later, cataract formation. Pike (5) discredited much of this work, especially with reference to the formation of a cataract. Although his rabbits showed, after repeated exposures to PDB vapors, some ocular pathology, he maintained that within seventeen days after the exposures had ceased all signs of eye pathology disappeared.

Ballotta and Mattioli (6) subjected rabbits to PDB vapors and claim that the blood picture showed only inconsiderable oscillation. No macroscopic or microscopic changes were perceptible in the various organs examined. They concluded that PDB vapors were harmless upon inhalation. Cameron, *et al.* (7), observed no significant changes in the organs of animals injected with PDB, but state that a slight liver necrosis may be produced. Teramoto (8) observed the effects of PDB vapors on guinea pigs, and noted that PDB caused only slight histological changes.

In view of the paucity of toxicological information concerning PDB and to the discrepancies observed in the literature, it appeared quite desirable to investigate further the toxic changes resulting from exposure to PDB.

EXPERIMENTAL

In all the ensuing experimental procedures pure PDB crystals "Mallinckrodt" (active ingredients 100%) were used.

* Received Aug. 11, 1948, from Purdue University, School of Pharmacy, Lafayette, Ind.
† Fellow of the American Foundation for Pharmaceutical Education.
‡ Professor of Pharmacology, Purdue University School of Pharmacy.
§ The authors are indebted to Dr. L. P. Doyle, Agriculture Experiment Station, Purdue University, for confirmation of pathologic findings.
[1] Hereafter designated as PDB.

I. Determination of the LD_{50} Dose

According to Thienes (9) no definite information is at hand as to the acute toxic dose of PDB; therefore, a determination of the LD_{50} dose was undertaken. Eight groups of ten albino (Wistar strain) rats, 50% male and 50% female, weighing between 125 to 275 Gm., maintained at a temperature 22 ± 1° and fasted overnight, were injected intraperitoneally with a solution of PDB in peanut oil (200 mg. per cc.), and observed for a 96-hour period. Doses ranged from 2000 to 3400 mg./Kg. The results are shown in Table I. The median lethal dose (LD_{50}), calculated by the method by Bliss (10), was found to be 2.562 ± S.E. 0.0125 Gm./Kg.

TABLE I.—RESULTS OF INTRAPERITONEAL INJECTIONS OF *p*-DICHLOROBENZENE IN PEANUT OIL INTO ADULT ALBINO RATS (WISTAR STRAIN)

Dose, Mg./Kg.	18 Hours Mortality	%	24 Hours Mortality	%	36 Hours Mortality	%[a]
2000	2/10	20	2/10	20	2/10	20
2200	3/10	30	3/10	30	4/10	40
2400	2/10	20	2/10	20	4/10	40
2600	2/10	20	3/10	30	4/10	40
2800	5/10	50	5/10	50	5/10	50
3000	6/10	60	6/10	60	6/10	60
3200	7/10	70	7/10	70	7/10	70
3400	5/10	50	5/10	50	7/10	70

[a] 48-, 72-, and 96-hour readings were the same as that recorded for 36 hours.

II. The Hematology of Warm-Blooded Animals Subjected to PDB Vapor Inhalation

A large chamber (102 x 45 x 46 cm.) with a capacity of 217.43 liters of air was constructed of one-quarter inch plywood. The interior was partitioned into three compartments by means of wire mesh and wooden slats. A hot plate with a piece of flat sheet metal on it was installed in the center compartment, the outer compartments being used for animals.

A calculated amount of PDB (21.7 Gm. equivalent to 100 mg. per liter of air) was weighed out, placed on the piece of sheet metal over the hot plate, and allowed to vaporize. Prior to this the animals were placed in the outer compartments and the chamber closed via a hinged outer door. The chamber was made further airtight by sealing with adhesive tape. The animals were exposed to the PDB vapors for a specified time in the chamber daily. An additional three minutes was allowed for the PDB crystals to vaporize. The temperature of the chamber was maintained at about 39–42°.

Three species of animals were used: rabbits, rats, and guinea pigs.

A. Rabbits.—A group of 18 male rabbits was used. The normal blood picture (white blood cell, red blood cell, and differential counts) and weight of each rabbit were determined before the first exposure. An additional rabbit was used as a control, making a total of 19 rabbits. All the rabbits, except the control, were exposed to PDB vapors in the chamber for a period of thirty minutes daily at an approximate concentration of 100 mg. PDB/liter of air. The control rabbit was placed in the chamber for the same period daily but without PDB vapors. At intervals of five to nine days the blood picture of each rabbit was noted, the marginal ear vein being the site of withdrawal of blood. An individual chart was kept for each animal and changes in weight and blood picture noted at intervals.

Resulting symptoms of exposure ranged from simple eye and nose irritation to the more usually encountered syndrome, viz., intense irritation of the eyes and nares, a pronounced "mark time" reflex, muscle twitches, tremors, loss of righting reflex, definite horizontal or vertical nystagmus, rapid but labored breathing. Recovery from symptoms ensued in from thirty minutes to two hours, depending upon the physiological state of the animal. The use of the hind legs was always the last function returning to normalcy.

A sample of the type of chart maintained is presented below (see Rabbit No. 1).

There was evidence of a granulocytopenia. Exposures ceased after the thirty-first day; blood picture was followed for return to normalcy. Twenty-seven days for normalization were needed.

A summarization of the effects of PDB vapors on rabbits after a series of exposures is given in Table II.

As seen in the Table, 11 rabbits exhibited a definite granulocytopenia, 3 were questionable, and 3 showed no decrease in the granulocytes (excluding the control). The weight decreased in 14 animals while only 3 showed an increase. An increase in total WBC's and RBC's was seen in 5 animals and a decrease in 12. The granulocytopenia and weight decrease can be considered as significant while the changes in total WBC and RBC counts are comparatively insignificant.

The 5 rabbits which were followed up after exposures ceased showed a normalization on the blood picture within thirty days.

RABBIT No. 1

No. of Exposure	Normal	8th Day	15th Day	20th Day	29th Day
Differential, %					
Neutrophils	29	31	13	12	12
Eosinophils	1	0	0	2	3
Basophils	0	0	0	0	0
Myelocytes	3	0	1	1	2
Lymphocytes	62	66	84	82	79
Monocytes	4	3	2	3	4
Unknown	1	0	0	0	0
White Blood Cell Count	6,850	8,300	5,100	6,600	5,250
Red Blood Cell Count	5,350,000	5,450,000	5,610,000	5,260,000	4,970,000
Weight, Kg.	1.87	1.89	1.81	1.73	1.59

B. Rats.—A group of 20 albino (Wistar strain) rats, 50% male and 50% female, ranging in weight from 150 to 300 Gm., maintained at a temperature of 22 ± 1° and fasted overnight, was used in this experiment; 18 were subjected to PDB vapors for a period of twenty minutes daily while 2 were used as controls.

The resultant effects of exposure to PDB vapors shown by the rats were remarkably similar to those shown by the rabbits. After each exposure there was complete narcosis with attendant tremors and twitches of the extremities. This condition usually lasted from one-half to three hours.

As with the rabbits, individual charts were maintained for each rat and changes in the blood picture carefully noted. A summarization of the effects of PDB vapors on rats after a series of exposures is presented in Table III.

Eight rats exhibited a definite granulocytopenia, 3 were questionable, and 2 showed no evidence of a lowering of granulocytes. Nine rats showed a decrease in total WBC's while 4 showed an increase. The granulocytopenia and the tendency toward this condition seem to be quite significant.

C. Guinea Pigs.—The same experimental conditions as described for the rabbits and rats were employed. A group of 9 male guinea pigs was used in this experiment and subjected to PDB vapors for twenty minutes daily.

Only differential cell counts were done since the total white blood cell and red blood cell counts showed no great variation in previous experiments with other warm-blooded animals.

The guinea pigs exhibited much the same symptoms as did the rabbits and rats, the symptoms lasting about one-half to one and one-half hours. As with the rabbits and rats, individual charts were maintained for each guinea pig and hematological and weight changes carefully noted.

Repeated exposures to this concentration (100 mg. of PDB per liter of air) produced such a marked intoxication in the guinea pigs that many of them died after only comparatively few exposures. A summarization of the effects of PDB vapors on guinea pigs is presented in Table IV.

Five of the guinea pigs exhibited granulocytopenia, 2 showed a tendency toward lowering, while 2 showed no decrease in granulocytes. Six of the animals showed a weight decrease while 3 showed none. The tendency toward granulocytopenia in this group of animals is apparent and significant. Two-thirds of the animals showed a decrease in weight. The guinea pig does not tolerate PDB vapors well since only 1 of the 9 test animals survived twenty exposures.

III. Histological Study

Thirteen rabbits, 15 rats, and 8 guinea pigs used in the hematological study previously described, and representing those animals dying or sacrificed after a series of exposures to PDB vapors, were autopsied and gross and microscopical changes in certain organs noted. The lungs, liver, kidney, and heart were studied in greatest detail. Each of these organs was carefully examined for gross pathology, and a section of tissue was taken from each to be used for study of microscopic changes.

The following procedure was used for making and staining the sections.

A 5-mm. piece of the tissue was placed in Bouin's solution and allowed to remain there overnight. The following day the tissue underwent a dehydration process through treatment with the following mixtures for the period of time specified:

1. 75% ethyl alcohol and 25% water for two hours.
2. 60% ethyl alcohol and 40% water for six hours.
3. 50% ethyl alcohol, 20% water, 30% *n*-butyl alcohol for six hours.
4. 50% ethyl alcohol, 15% water, 35% *n*-butyl alcohol for six hours.
5. 35% ethyl alcohol, 5% water, 60% *n*-butyl alcohol for six hours.
6. 30% ethyl alcohol, 70% *n*-butyl alcohol for six hours.
7. 100% *n*-butyl alcohol for six hours (first change).
8. 100% *n*-butyl alcohol for six hours (second change).

TABLE II.—A SUMMARIZATION OF THE EFFECTS OF *p*-DICHLOROBENZENE VAPORS ON RABBITS

Rabbit No.	% Change in Granulocytes	No. of Exposures	Death or Follow-up	Change in Weight, Kg.	WBC Total	RBC Total
1	33–17	31	Follow-up	1.87–1.59	Decrease	Decrease
2	31–19	32	Follow-up	1.78–1.56	Increase	Increase
3	29–25	33	Death	2.53–2.27	Decrease	Decrease
4	28–19	33	Death	2.55–2.51	Decrease	Decrease
5	41–29	15	Death	1.60–1.71	Decrease	Increase
6	29–18	16	Death	2.20–1.74	Increase	Decrease
7	41–18	26	Death	1.69–1.39	Decrease	Decrease
8	36–33	29	Death	1.73–1.74	Decrease	Decrease
9	28–25	16	Death	2.10–2.08	Increase	Decrease
10	38–29	11	Death	2.12–2.19	Decrease	Increase
12[a]	43–29	12	Death	1.83–1.67	Decrease	Decrease
13	40–20	31	Follow-up	3.07–2.61	Decrease	Decrease
14	44–22	30	Death	2.33–1.85	Decrease	Decrease
15	31–20	31	Death	2.93–2.92	Decrease	Decrease
16	42–28	32	Follow-up	3.58–3.14	Increase	Increase
17	52–17	33	Follow-up	4.55–4.09	Decrease	Decrease
18	60–29	34	Death	3.41–2.93	Increase	Increase
19	34–35	30	Control	2.78–3.29	Increase	Decrease

[a] Rabbit 11 died after the first exposure and is omitted.

TABLE III.—A SUMMARIZATION OF THE EFFECTS OF *p*-DICHLOROBENZENE VAPORS ON RATS

Rat No.	% Change in Granulocytes	No. of Exposures	WBC Total	RBC Total
1	26–14	25	Decrease	Decrease
2	32–17	25	Decrease	Increase
3	22–18	25	Increase	Increase
4	36–37	25	Increase	Increase
5	40–33	11	Decrease	Increase
6	21–11	22	Decrease	Increase
7	36–16	22	Increase	Increase
8	34–17	22	Decrease	Decrease
9	37–16	22	Decrease	Decrease
10	31–18	22	Decrease	Decrease
11[a]	19–33	22	Decrease	Increase
15[b]	20–11	10	Decrease	Decrease
18[c]	17–14	18	Increase	Increase

[a,b] Nos. 12, 13, 14, 16, and 17 died after a few exposures and are therefore eliminated from the above table.
[c] Nos. 19 and 20 (controls) showed little deviation from normal.

TABLE IV.—A SUMMARIZATION OF THE EFFECTS OF *p*-DICHLOROBENZENE VAPORS ON GUINEA PIGS

Guinea Pig No.	% Change in Granulocytes	No. of Exposures	Change in Weight, Kg.
1	36–29	8	0.625–0.618
2	31–20	16	0.555–0.532
3	46–29	17	0.675–0.661
4	39–26	14	0.590–0.582
5	36–25	12	0.690–0.678
6	29–30	6	0.660–0.662
7	29–32	15	0.605–0.619
8	35–21	20	0.555–0.530
9	43–37	6	0.800–0.802

After dehydration the tissue was imbedded in "mush paraffin," a mixture of 20% *n*-butyl alcohol and 80% paraffin (Bioloid, m. p. 53–55°).

The tissue remained imbedded for a minimum of twelve hours, after which time transfer to pure paraffin was effected. After twelve hours in the pure paraffin, the tissue was ready for blocking.

A porcelain or glass boat of a suitable size was coated liberally with glycerin and pure melted paraffin poured into the container. The tissue was transferred into this container and the paraffin allowed to cool and harden. After complete hardening the block was loosened from the container and cut into rectangular or square sections to be fitted onto the pivot of the microtome.

A small amount of wax was built up on the pivot, heated slightly, and the section of imbedded tissue pressed upon the pivot which was then inserted into the microtome, ready for cutting. All sections were cut at a 10-μ thickness. A warm stage maintained at about 40° was brought into use. Glass slides with a drop of distilled water on each were placed on this stage and the cut sections laid flattened out on the slides. This greatly facilitated adherence of the cut section to the glass slide. The cut sections were allowed to remain on the warm stage overnight to insure further adhesiveness to the slide. The following morning the sections were ready for staining.

The slides were placed back to back in a staining dish carrier and immersed into each of the following solutions for a specified time:

1. Xylol—two to three minutes to dissolve the paraffin.
2. Xylol—two to three minutes, second change.
3. *n*-Butyl alcohol—two minutes.
4. Ethyl alcohol—two minutes.
5. 80% alcohol—two minutes.
6. Distilled water rinse—one minute.
7. Hematoxylin stain—twenty minutes.
8. Rinse thoroughly in tap water, removing all stain excess.
9. Immerse in acid-alcohol, with care, to decolorize.
10. Rinse thoroughly in tap water.
11. Eosin stain—three to five minutes.
12. Rinse well in tap water.
13. 70% alcohol—two minutes.
14. Ethyl alcohol—two minutes.
15. *n*-Butyl alcohol—one minute, first change.
16. *n*-Butyl alcohol—one minute, second change.
17. Xylol—two minutes, first change.
18. Xylol—until ready for mounting.

The slides were removed from the xylol and mounts made with either Clarite or Demar and covered with a cover glass. The slides were then allowed to dry overnight on the warm stage, being ready for microscopic examination the following morning.

Several sections of each organ from each animal were made and all deviations from the normal histological appearance carefully noted. The rabbits showed varying pathological changes and are therefore reported individually. However, the rats and guinea pigs as a group showed identical changes and consequently are reported as a group only.

A. Rabbits.—Twelve rabbits that died of exposure to PDB vapors during the hematological study previously described and one control rabbit were autopsied and sectioned. A typical report of gross and microscopic findings is presented. The results for Rabbit No. 3 are as follows:

Gross Pathology.—The left lung showed hemorrhagic areas; other parts of the lung appeared normal. The lungs were colored a dark red. The liver and heart appeared to be normal. The kidneys showed no visible changes except for a slightly swollen appearance.

Microscopic Pathology.—The lungs were markedly hyperemic and edematous with fresh hemorrhages in the bronchi and alveoli and large amounts of serous exudate in the bronchi. (See Fig. 1.) Atelectasis was very prominent along with occasional emphysemic areas. The microscopic findings preclude true pneumonia as the possible cause of death. The lymphoid tissue showed no hypertrophy; nuclei showed shrinkage and condensation (pyknosis).

The kidneys showed evidence of much damage characterized by the swollen epithelium of the uriniferous tubules, especially of the proximal and distal convoluted tubules. Epithelium of the tubules showed early albuminous degenerative changes. (See Fig. 2.) The nuclei of the cells showed loss of alignment and were arranged in a disorderly fashion. Many cellular casts were present in the collecting tubules. (See Fig. 3.) The glomeruli exhibited a definite hyperemia.

There was no apparent damage to the liver cells themselves. Many edematous and hyperemic areas

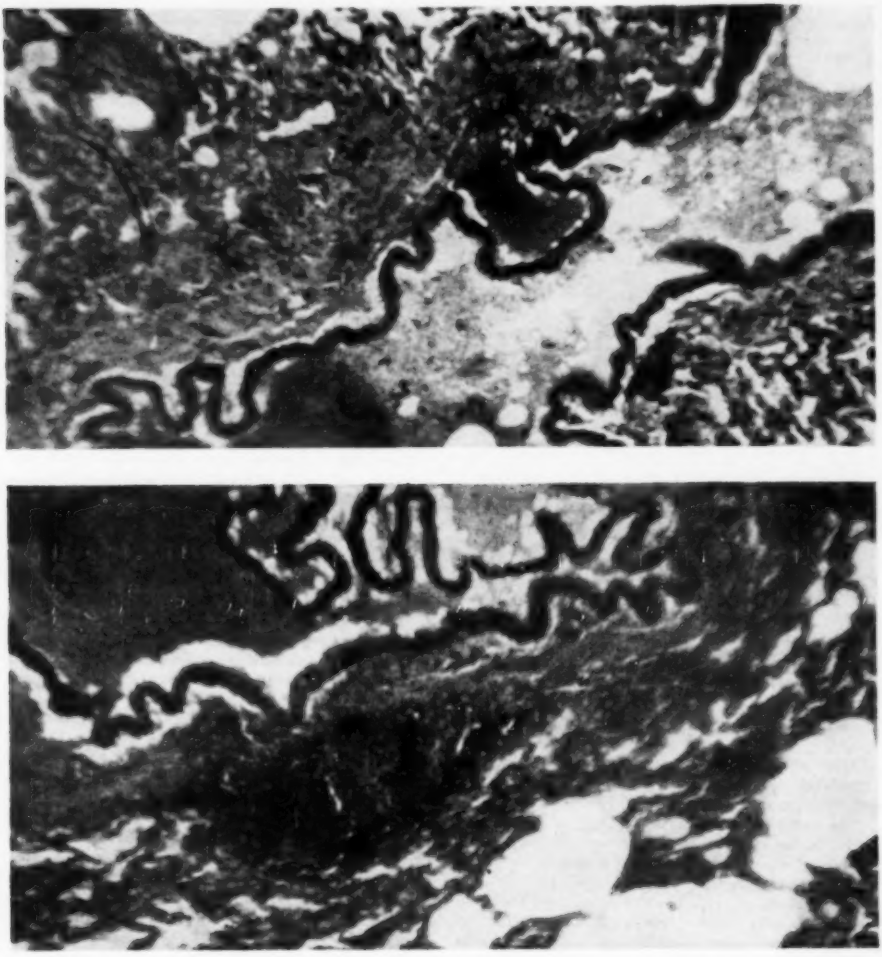


Fig. 1.—Photomicrographs showing serous exudate in bronchi of lung and loosening of the epithelium (rabbit).

NOTE: Many sections showed accumulations of serous fluid in the alveoli (rabbit, guinea pig, and rat).

were seen with an occasional lobule congested and the adjoining sinusoid space filled with blood. There was no evidence to indicate a definite hepatitis.

The heart showed no obvious damage. The nuclei were intact, there was some separation of muscle fiber, and occasionally a possible degenerative change in the muscle noted by the lack of striations in the muscle. However, the latter possibly could be due to fixing technique.

B. Rats.—Thirteen rats, sacrificed after a series of exposures to PDB vapors during the hematological study previously described, and two control rats were autopsied and sectioned. All the rats showed somewhat the same type of gross and microscopic pathology and are therefore presented as a group in the following report:

Gross Pathology.—All the rats, except the controls which appeared to be normal, exhibited hemorrhagic areas in the lungs of varying degrees as well as a very bright reddish color to either one or both lungs. The heart was of normal size and color in all animals. The majority showed no liver abnormality with the exception of a few which exhibited white streaks on the surface. The kidneys appeared to be normal except for a few that were swollen.

Microscopic Pathology.—The animals showed little variation of histological change. Hyperemic and edematous areas were most frequently encountered

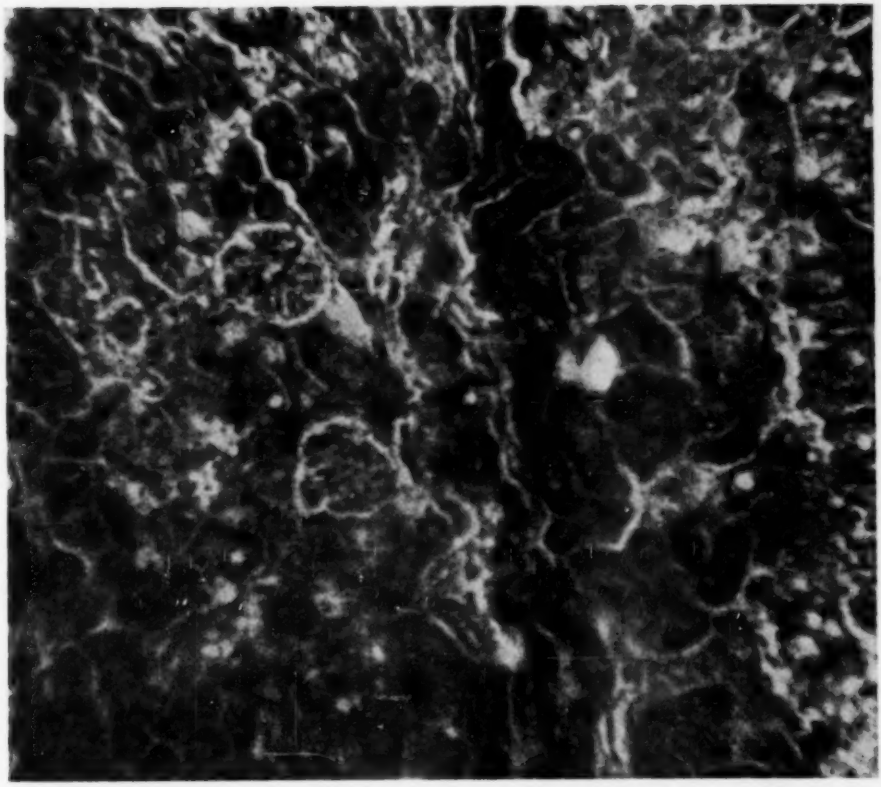


Fig. 2.—A photomicrograph of a rabbit kidney showing marked degenerative changes in the epithelium of the convoluted tubules. The glomeruli exhibit swelling of the tufts and pyknosis of the cells in the tufts.

in the lungs. The epithelium of the bronchi did not appear to be much changed in most cases. Occasionally a dissociation of the epithelium of the bronchi was seen but never very clearly. Fresh hemorrhages in the lungs were observed, mainly in the bronchi. No hypertrophy of the lymphoid tissue was noted. A pyknotic appearance of the nuclei was common. Neither damage to the cells nor a hepatitis was observed in the liver sections of the rats. Some hyperemic and edematous areas were seen. All the rats showed extensive damage to the kidneys. The epithelium of the tubules was swollen in most cases. The glomeruli were swollen and hyperemic with very marked pyknosis. Almost all the tubules showed varying degrees of necrobiosis. Some animals showed degeneration and loss of alignment of the nuclei. A few of the animals showed a granular exudate in the collecting tubules. Generally the damage to the kidneys was severe. No definite heart damage was observed. Occasionally an edematous area was noted but the nuclei and striation were intact and visible.

C. Guinea Pigs.—Eight guinea pigs were autopsied and sectioned and are presented as a group since the findings were not too variable.

Gross Pathology.—The lungs exhibited very dark hemorrhagic areas although the color of the lungs did not vary greatly from the normal. The kidneys did not appear to be swollen, but showed reddish dotted areas on the surface. Many of the animals had a white streak on the liver surface. The heart showed no visible changes.

Microscopic Pathology.—The lungs were, in general, markedly congested and edematous. Distention of the alveoli was a prominent feature. Dissociation and loosening of the epithelium of the bronchi were prominently observed. Emphysematous areas were encountered frequently. Some bronchi were filled with serous exudate and with blood from fresh hemorrhages. All the animals showed extensive kidney damage in the form of swollen as well as degenerated epithelium of the uriniferous tubules. The glomeruli were swollen and congested and pyknosis was much in evidence. No evidence of a definite hepatitis or cellular damage to the liver was observed. A few edematous and hyperemic areas were seen in the liver of some animals. The nuclei of the heart were intact. Occasionally an animal would exhibit some early changes in the form of an infiltration which was

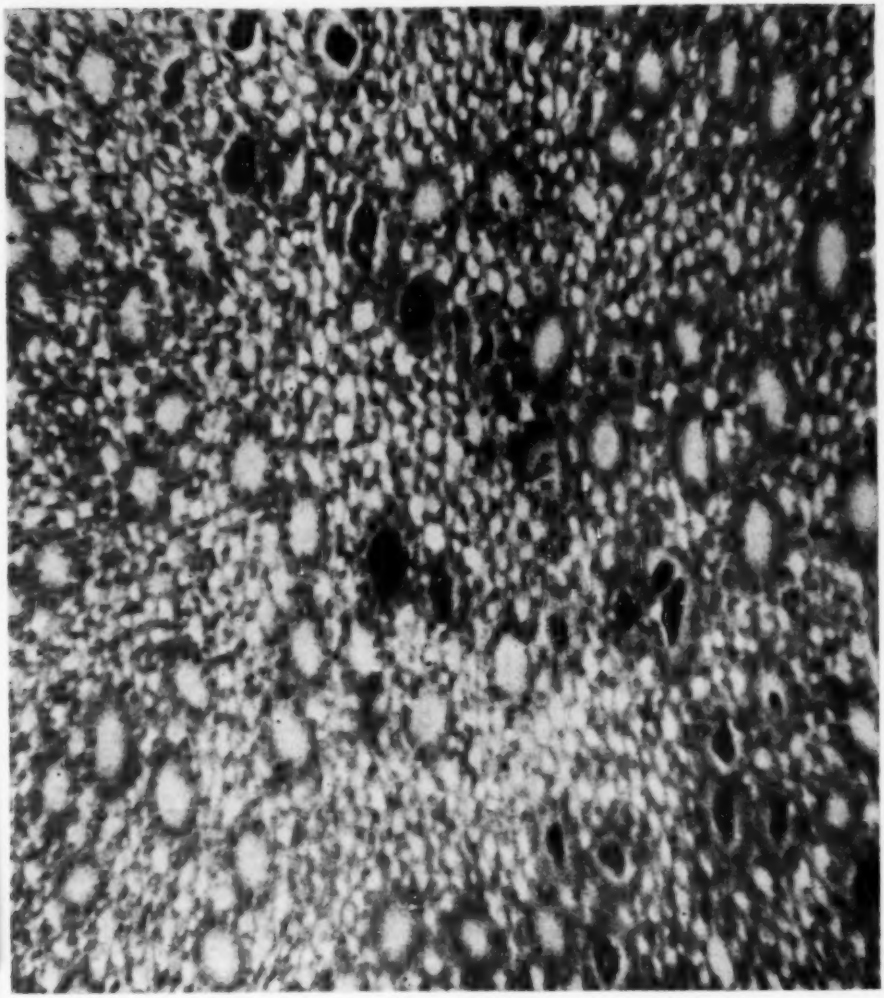


Fig. 3.—A photomicrograph of cellular casts in straight collecting tubules of rabbit kidney.

characterized by round cells—histiocytes and monocytes—indicating a possible myocarditis. In general, however, little or no pathology of the heart tissue was encountered.

SUMMARY

1. A determination of the LD_{50} dose of *p*-dichlorobenzene in peanut oil, via intraperitoneal injection, using albino (Wistar strain) rats has been presented. According to Bliss, this was found to be 2.562 ± S. E. 0.0125 Gm./Kg.

2. Inhalation of PDB vapors in sufficient concentration produced a marked intoxication in the rabbit, rat, and guinea pig characterized by a definite and constant syndrome. This syndrome consists of intense eye and nares irritation, tremors and twitches of the extremities, a "mark time" reflex, a loss of the righting reflex, a definite nystagmus, and rapid but labored respiration. Death may occur; if not, complete recovery ensues within one-half to two hours depending upon the physiological state of the animal.

3. The results of a hematological study of rabbits, rats, and guinea pigs after a series of exposures to PDB vapors indicate that this compound has a selective action on the granulocytes

of the blood, producing a granulocytopenia in a majority of the animals. The total leucocytic (showing some increase in lymphocytes) and erythrocytic counts were not greatly affected. Normalization of the blood picture occurred within thirty days after exposures were discontinued.

4. A histological study of twelve rabbits, thirteen rats, eight guinea pigs, representing those animals dying or sacrificed after a series of exposures to PDB vapors, revealed that PDB has a definite selective action on the kidneys. Every animal showed marked and extensive damage. Contrary to reports in the literature, comparatively little liver damage or evidence of a definite hepatitis was observed. There was the expected amount of damage to the lung tissue. Damage to the heart tissue was negligible.

5. Inhalation of PDB vapors produced severe toxic effects in small laboratory animals only if the concentration was unusually high and the vapors were inhaled over a sufficiently long period of time.

REFERENCES

(1) Shepard, Harold H., "The Chemistry and Toxicology of Insecticides," Burgess Publishing Co., Minneapolis, 1945, p. 343.

(2) Lindquist, A. W., and Wilson, H. G., Insect Control Committee Abstract Bulletin No. 2, p. 43, National Research Council, Feb. 18, 1946.

(3) Perrin, Maurice, *Bull. Acad. Med.*, **125**, 302(1941); *C. A.*, **37**, 3833(1943).

(4) Berliner, M. L., *Arch. Ophthalmol. (Chicago)*, **22**, 1023 (1939).

(5) Pike, M. H., *J. Michigan Med. Soc.*, **43**, 581(1944).

(6) Ballotta, F., and Mattioli, P., *Arch. Ital. Sci. Farmacol.*, **10**, 65(1941); *C. A.*, **37**, 2815(1943).

(7) Cameron, G. R., Thomas, J. C., Ashmore, S. A., Warren, E. M., Buchan, J. L., and Hughes, A. W. M., *J. Path. Bact.*, **44**, 281(1937).

(8) Teramoto, M., *Nagoya Jour. Med. Sci.*, **7** (2), 69 (1933); *Biol. Abstracts*, **10**, 13482(1936).

(9) Thienes, C. H., "Clinical Toxicology," ed. 1, Lea and Febiger, Philadelphia, 1940, p. 166.

(10) Bliss, C. I., *Quart. J. Pharm. Pharmacol.*, **11**, 192 (1938).

Standard Tolerances for Pharmaceutical Compounding. A Basis for Their Establishment. II. Liquid Preparations*

BY SAMUEL W. GOLDSTEIN†, ‡, §

The validity of the method described in this report for the determination of reasonable and equitable standards of tolerance for extemporaneously compounded pharmaceuticals rests primarily in the samples utilized. These samples were purchased from many practicing pharmacists who are possessed of many degrees of natural skill, and who work under the many different conditions existing in all types of licensed pharmacies. The indicated tolerances are obtained by statistical studies of different series of preparations utilizing the system of least squares and 1.73 standard deviations. The acceptable proportion of samples in each series as determined by this procedure is compared with the acceptable proportion of samples as determined by the points of flexure on probability curves plotted for each series. This comparison indicates that, at least in this type of work, the adopted method yields functionally true tolerances.

THE PROCEDURES employed in these studies and the method of sample collection were described in an earlier paper by the author (1). The data obtained with samples of liquid preparations that were prepared extemporaneously by many licensed pharmacists at their prescription counters are considered in this paper. Samples with deviations that are approximate multiples of the requested weight, or are divisions approximately one-third or more of such weight, have been excluded from consideration in the determination of limits of acceptance or tolerances. Practically all of these extreme deviations can be attributed to mathematical miscalculations, and they should not exert any influence in the determination of standards of tolerance for manipulative precision. The Standard Deviation (S. D.) is based upon devia-

* Received April 26, 1948, from the Bureau of Chemistry, Maryland State Department of Health, Baltimore, Md.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.

† Pharmaceutical Chemist, Maryland State Health Department, Baltimore 18, Md. Member of A. PH. A. Committee on Prescription Tolerances.

‡ The conclusions presented in this paper represent the personal opinions of the author.

§ The author is indebted to Mr. J. R. McComas for his assistance.

tions from the theoretical mean and is determined for each series of samples. The S. D. is converted to the equivalent Coefficient of Variation, which is multiplied by the ratio "1.73" to obtain the calculated tolerances for the different preparations. The data for each series are plotted as a "cumulative curve of deviation" and the points of flexure are found by bisection of the angles of tangential intersection. These points are used as comparative provisional limits of acceptance. The calculated tolerance is used in assigning the standard tolerance from a scale which increases by increments of 2.5.

EXPERIMENTAL

All the preparations were tested by the author, using official assays whenever this was possible. Some of the Chloroform Spirit samples were assayed by the procedure which later was adopted by the National Formulary.

The first series contains 191 "selected" samples of Potassium Iodide Solution N. F. (100% w/v), which were purchased in ½-ounce or 30-cc. quantities. The data are summarized in Table I. The calculated tolerance is ±8.65%, and allows acceptance of 90% of the 195 samples purchased. The points of flexure on the cumulative curve of deviation for this series (Fig. 1) allow acceptance of 89% of all the samples.

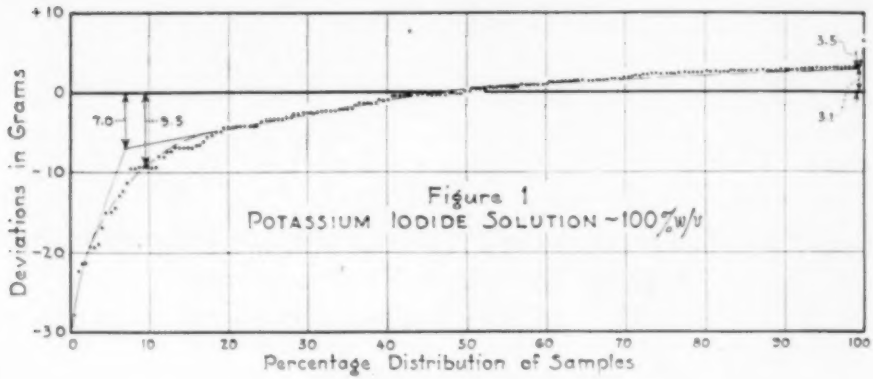


Figure 1
POTASSIUM IODIDE SOLUTION ~100% w/v

TABLE I.—SUMMARY OF ACCUMULATED DATA

Solution	No. of Samples Purchased	Lowest Concn., %	Concn. of Lower Quartile, %	Concn. of Median, %	Concn. of Upper Quartile, %	Highest Concn., %
Pot. Iodide 100%	195	72.00	96.60	99.60	102.30	106.60
Ammon. Chlor. 19.7%	77	16.22	18.86	19.31	19.70	20.81
Acetic Ac. 10%	64	4.00	9.70	10.20	10.46	13.71
Hydrochlor. Ac. 10%	388	3.64	9.46	9.96	10.36	15.11
Chlorof. Spt. 8.8%	55	4.33	8.28	8.54	8.72	10.65
Pot. Permang. 3%	102	1.00	2.90	3.00	3.10	3.53
Boric Ac. 3%	99	0.95	2.89	2.99	3.15	4.31
Pot. Permang. 2.5%	96	1.38	2.40	2.50	2.60	3.22
Phenol in Oil 10%	44	7.92	9.09	9.80	10.30	19.51
Pot. Permang. 0.5%	64	0.03	0.49	0.50	0.54	0.73
Silver Nitrate 1%	192	0.58	0.97	1.02	1.08	1.59
Phenol in Oil 2%	93	0.62	1.49	1.98	2.12	3.53

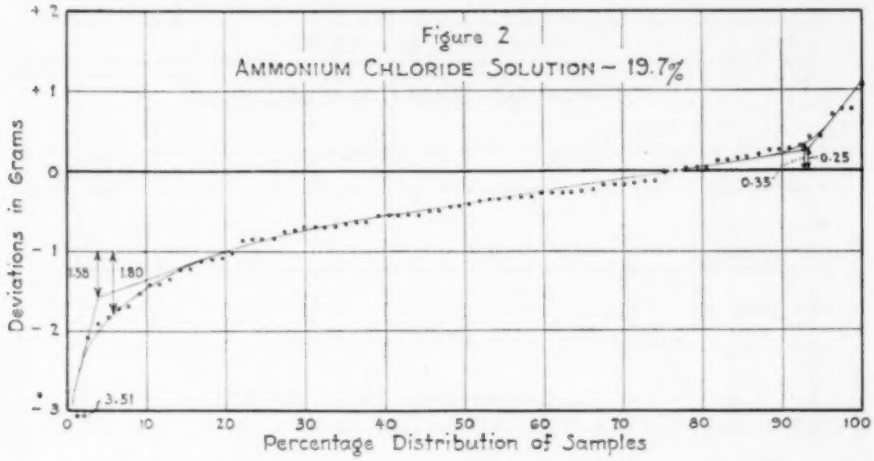
	No. of Samples Purchased	Greatest Minus Dev., %	Dev. of Lower Quartile %	Dev. of Median, %	Dev. of Upper Quartile, %	Greatest Plus Dev., %
Pot. Iodide 100%	195	−28.0	− 3.4	−0.4	+2.3	+ 6.6
Ammon. Chlor. 19.7%	77	−17.8	− 4.4	−2.1	0.0	+ 5.5
Acetic Ac. 10%	64	−60.0	− 3.0	+2.0	+4.6	+37.1
Hydrochlor. Ac. 10%	388	−63.6	− 5.4	−0.4	+3.6	+51.1
Chlorof. Spt. 8.8%	55	−50.8	− 5.9	−3.0	−0.9	+21.0
Pot. Permang. 3%	102	−66.7	− 3.3	0.0	+3.3	+17.7
Boric. Ac. 3%	99	−68.3	− 3.7	−0.3	+5.0	+43.7
Pot. Permang. 2.5%	96	−44.8	− 4.0	0.0	+4.0	+28.8
Phenol in Oil 10%	44	−20.8	− 9.1	−2.0	+3.0	+95.1
Pot. Permang. 0.5%	64	−94.0	− 2.0	0.0	+8.0	+46.0
Silver Nitrate 1%	192	−42.0	− 3.0	+2.0	+8.0	+59.0
Phenol in Oil 2%	93	−69.0	−25.5	−2.0	+6.0	+76.5

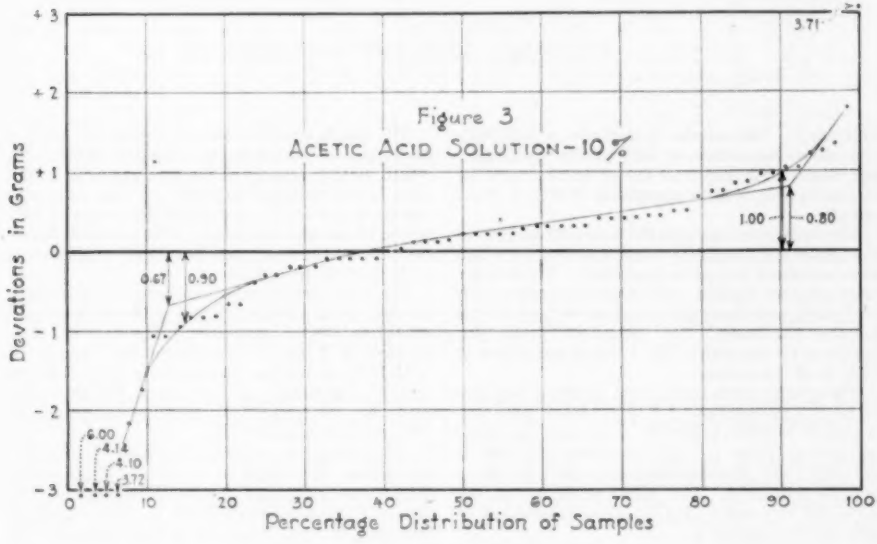
The second series contains 77 samples of Ammonium Chloride Solution, which were requested as: Ammon. Chlor. dr. 4½, Water *q. s.* 3 oz. This represents the simplest type of liquid preparation. No calculation is required and a large quantity of the ingredient must be weighed. This is reflected in the fact that no sample in this series was rejected on the basis of excessive deviation. The data are summarized in Table I. The calculated tolerance is ±7.82%, and allows acceptance of 93% of the samples. The points of flexure on the curve for this series (Fig. 2) allow acceptance of 87% of all the samples.

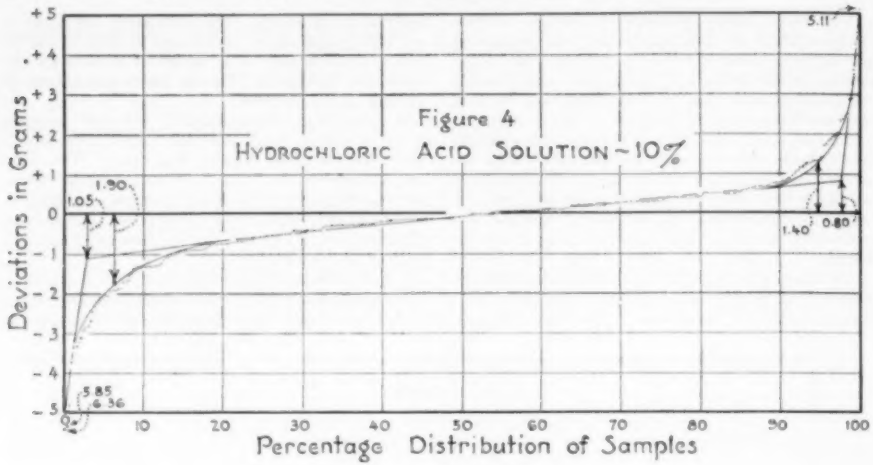
The third series contains 59 "selected" samples of Acetic Acid Solution (10% w/v), which were purchased in 2-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±14.54%, and allows acceptance of 88% of the 64 samples purchased. The points of flexure on the curve for this series (Fig. 3) allow acceptance of 77% of all the samples.

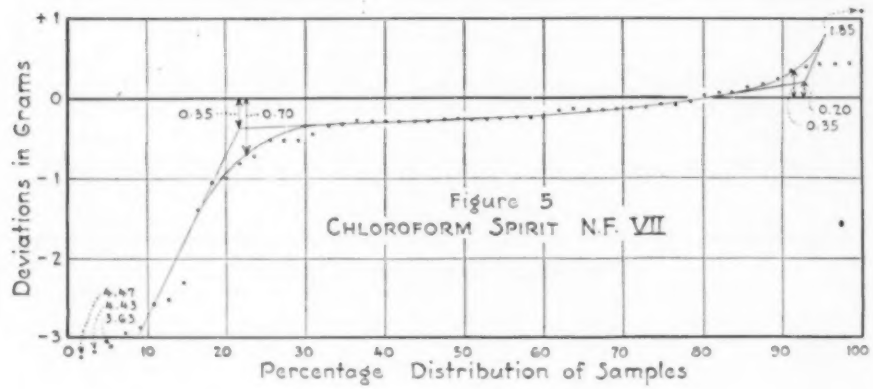
The fourth series contains 377 "selected" samples of Hydrochloric Acid Solution (10% w/v), which were purchased in 2-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±15.74%, and allows acceptance of 87% of the 388 samples purchased. The points of flexure on the curve for this series (Fig. 4) allow acceptance of 88% of all the samples.

The fifth series contains 50 "selected" samples of Chloroform Spirit N. F. VII, which were purchased in 2-ounce quantities. The data are summarized









in Table I. The calculated tolerance is ±15.34%, and allows acceptance of 82% of the 55 samples purchased. The points of flexure on the curve for this series (Fig. 5) allow acceptance of 67% of all the samples.

The sixth series contains 95 "selected" samples of Potassium Permanganate Solution (3% *w/v*), which were purchased in 3-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±11.02%, and allows acceptance of 86% of the 102 samples purchased. The points of flexure on the curve for this series (Fig. 6) allow acceptance of 73% of all the samples.

The seventh series contains 90 "selected" samples of Boric Acid Solution (3% *w/v*), which were purchased in 3-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±11.70%, and allows acceptance of 86% of the 99 samples purchased. The points of flexure on the curve for this series (Fig. 7) allow acceptance of 83% of all the samples.

The eighth series contains 94 "selected" samples of Potassium Permanganate Solution (2.5% *w/v*), which were purchased in 3-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±14.0%, and allows acceptance of 88% of the 96 samples purchased. The points of flexure on the curve for this series (Fig. 8) allow acceptance of 81% of all the samples.

The ninth series contains 42 "selected" samples of Phenol in Oil Solution (10% *w/v*), which were purchased in 1-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±15.76%, and allows acceptance of 87% of the 44 samples purchased. The points of flexure on the curve for this series (Fig. 9) allow acceptance of 84% of all the samples.

The tenth series contains 62 "selected" samples of Potassium Permanganate Solution (0.5% *w/v*), which were purchased in 3-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±16.63%, and allows acceptance of

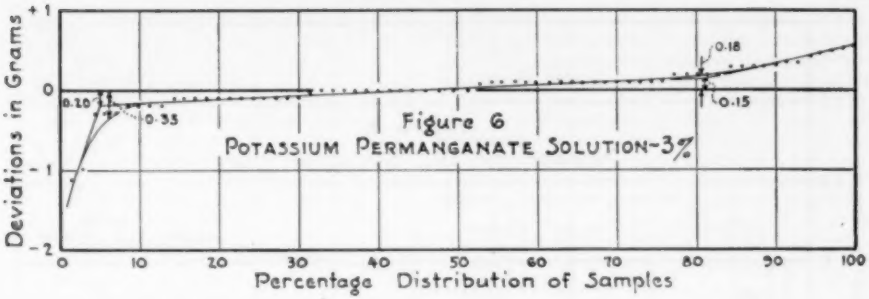


Figure 6
POTASSIUM PERMANGANATE SOLUTION-3%

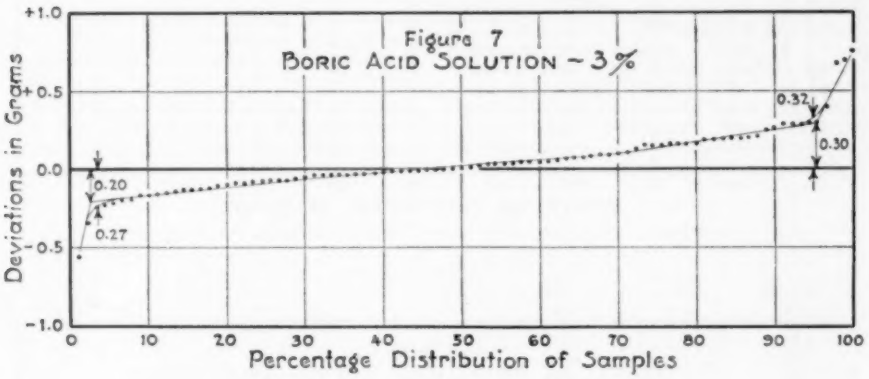


Figure 7
BORIC ACID SOLUTION ~ 3%

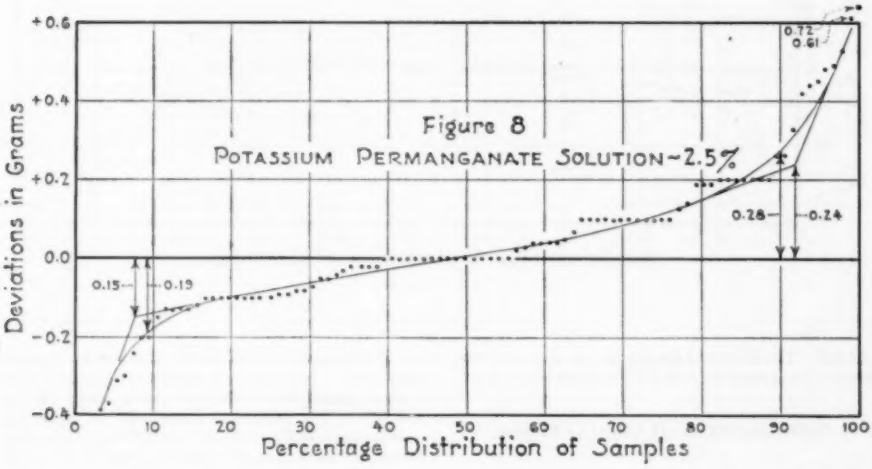
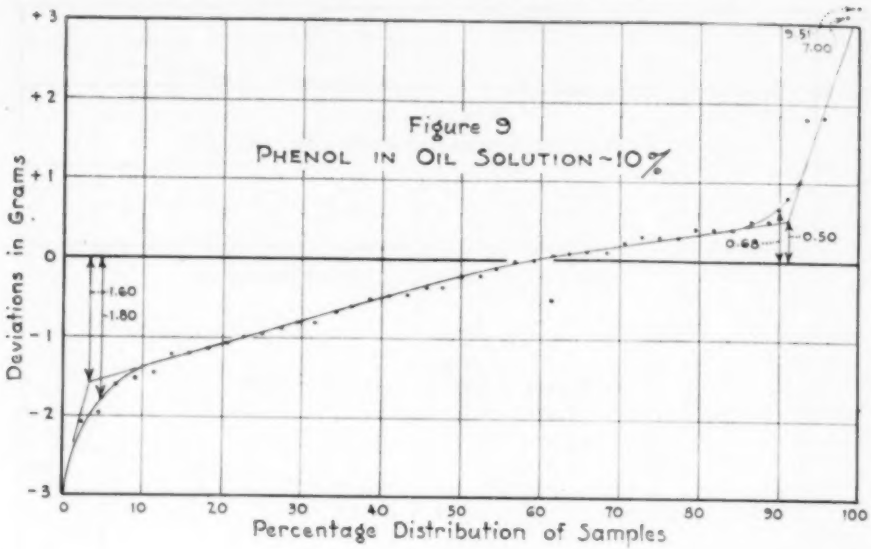


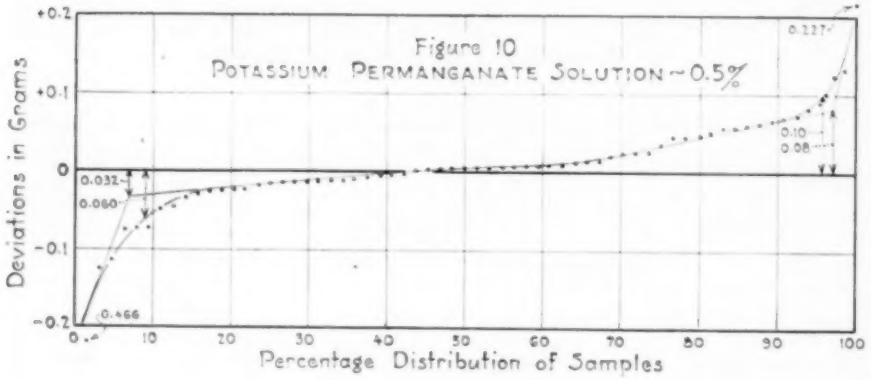
Figure 8
POTASSIUM PERMANGANATE SOLUTION~2.5%

89% of the 64 samples purchased. The points of flexure on the curve for this series allow acceptance of 86% of all the samples.

The eleventh series contains 189 "selected" samples of Silver Nitrate Solution (1% *w/v*), which were purchased in 2-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±15.65%, and allows acceptance of 91% of the 192 samples purchased. The points of flexure on the curve for this series allow acceptance of 92% of all the samples.

The twelfth series contains 69 "selected" samples of Phenol in Oil (2% *w/v*), which were purchased in 1-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±19.26%, and allows acceptance of 66% of the 93 samples pur-





chased. The points of flexure on the curve for this series allow acceptance of 80% of all the samples.

DISCUSSION AND CONCLUSIONS

Under the conditions of these studies, 1.73 standard deviations yield tolerances which appear to represent reasonable and equitable limits of deviation in the compounding of extemporaneous preparations at the prescription counter. These values can be used in establishing standards of tolerance.

It is an undisputed fact that even under controlled conditions, the probability for error increases as the amount of material used decreases. This probability is increased further under prescription-counter conditions. For this reason the solutions are arranged according to the decreasing order of the weights of the medicinal ingredients in Table II.

It is apparent that another factor has disturbed the corresponding increasing order of error as indicated by the determined tolerances. It is also apparent that the solutions which show deviations higher than are indicated by their relative positions, possess the common factor of having an unstable and/or volatile ingredient, such as a volatile acid, chloroform, or phenol. Using the criteria of weight and nature of ingredients, the following classification of liquid preparations is offered for the purpose of establishing standard tolerances.

Group 1. Total weight of ingredient 5 Gm. or more.
- (*a*) Stable ingredients.
- (*b*) Unstable ingredients.

Group 2. Total weight of ingredient not less than 2.25 Gm. nor more than 4.99 Gm.
- (*a*) Stable ingredients.
- (*b*) Unstable ingredients.

Group 3. Total weight of ingredient not less than 0.5 Gm. nor more than 2.24 Gm.
- (*a*) Stable ingredients.
- (*b*) Unstable ingredients.

Group 4. Total weight of ingredient 0.49 Gm. or less.
- (*a*) Stable ingredients.
- (*b*) Unstable ingredients.

When more than one ingredient is present in a preparation, the total weight and nature of each ingredient shall determine its individual classification.

It is obvious that studies of different preparations placed in the same group and division would yield identical calculated tolerances only by coincidence. A standard tolerance is therefore assigned to each preparation from a scale increasing by increments of ±2.5%. The tolerances assigned to the different groups are derived from the standard tolerances assigned to preparations which unmistakably belong in each particular division. The group assignments and the determined tolerances of the studied solutions are shown in Table II. Using the scale-assigned tolerances for the preparations, the following tentative group tolerances are obtained.

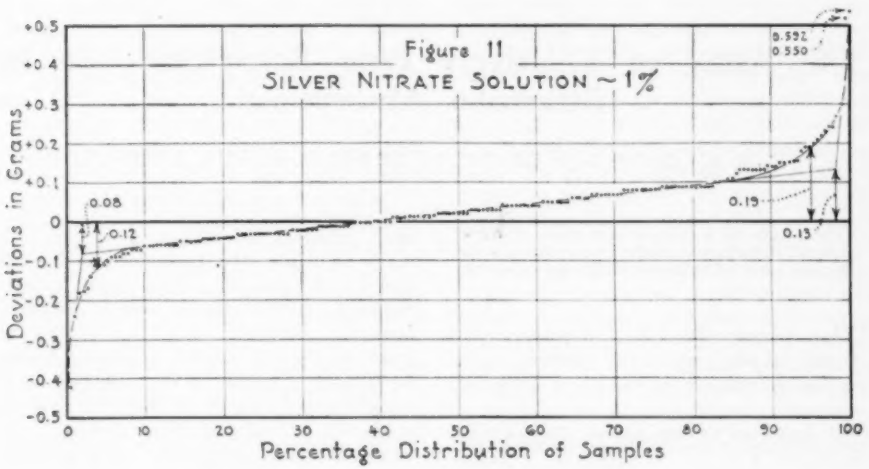
Group 1. (*a*) ±10.0%; (*b*) ±15.0%.
Group 2. (*a*) ±12.5%; (*b*) ±17.5%.
Group 3. (*a*) ±15.0%; (*b*) ±20.0%.
Group 4. (*a*) ±17.5%.

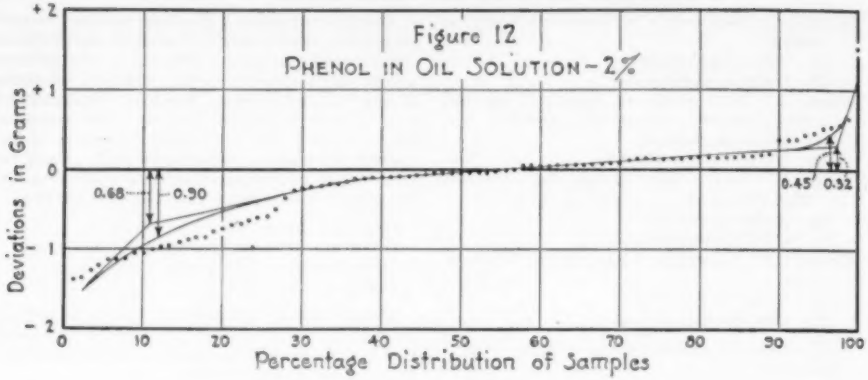
These Standard Tolerances can be applied to any unstandardized preparation containing ingredients that can be accurately determined and are assignable to one of the group divisions.

There is no doubt that further study on a collaborative basis is desirable before standard tolerances for extemporaneously compounded liquid preparations can be officially promulgated. The method presented here appears to yield reasonable and equitable

TABLE II.—CLASSIFICATION AND TOLERANCES OF LIQUID PREPARATIONS

No. of Selected Samples	Solution	Wt. of Ingredient Requested, Gm.	Group Placement	Calcd. Tolerance, ±%	Assigned Tolerance, ±%
77	Ammon. Chlor. 19.7%	17.50	1 (*a*)	7.82	7.5
191	Pot. Iodide N. F.	15.00	1 (*a*)	8.65	10.0
59	Acetic Ac. 10%	5.91	1 (*b*)	14.54	15.0
377	Hydrochlor. Ac. 10%	5.91	1 (*b*)	15.74	15.0
50	Chlorof. Spt. N. F.	5.32	1 (*b*)	15.34	15.0
42	Phenol in Oil 10%	2.96	2 (*b*)	15.76	17.5
95	Pot. Permang. 3%	2.66	2 (*a*)	11.02	12.5
90	Boric Ac. 3%	2.66	2 (*a*)	11.70	12.5
94	Pot. Permang. 2.5%	2.22	3 (*a*)	14.00	15.0
190	Silver Nitrate 1%	0.59	3 (*a*)	15.65	15.0
69	Phenol in Oil 2%	0.59	3 (*b*)	19.26	20.0
62	Pot Permang. 0.5%	0.44	4 (*a*)	16.63	17.5





tolerances, and it is recommended that it be used as a basis for the establishment of such standards. These tentative standards are also recommended to the Food and Drug Officials of the individual states as an aid in judging the quality of prescription-counter products on a reasonable and equitable basis.

SUMMARY

1. Tolerances are determined for twelve liquid preparations by means of the Standard Deviation based on the least squares. The percentages of the samples acceptable by this method are compared with the values determined by the points of flexure on serial curves of deviation.

2. The Coefficient of Variation equivalent to 1.73 Standard Deviations of an adequate series of samples is recommended as a reasonable and equitable tolerance.

3. A system of classification of liquid preparations based upon the weight and nature of the ingredients is described.

4. A system for assigning Standard Tolerances from the determined tolerances, and the application of these Standard Tolerances to any preparation meeting the classification requirements are described.

5. Tentative Standard Tolerances have been determined for the different classifications.

6. It is recommended that the tentative Standard Tolerances should be used by Food and Drug Officials as a reasonable and equitable basis for their decisions.

REFERENCES

(1) Goldstein, S. W., THIS JOURNAL, **38**, 18(1949).

The Assay of Relatively Pure Benzylpenicillin by Ultraviolet Absorption*

By A. A. COLON, G. E. HERPICH, J. D. NEUSS, and H. A. FREDIANI

The results of a comparative study of three spectrophotometric methods currently in use for the assay of benzylpenicillin are reported.

THE CURRENT official F. D. A. method (1) is still the sole, truly specific chemical method for the quantitative estimation of benzylpenicillin (penicillin G). This method, reported in detail by Mader and Buck (2) and Sheehan, Mader, and Cram (3), is least subject to interference by other penicillins and by extraneous materials. However, since this method requires two to four hours per analysis, a more rapid screening method for relatively high purity material is desirable for routine controls in the production of salts of benzylpenicillin. The fortuitous presence of a phenyl group in benzyl-

* Received Sept. 29, 1948, from the Chemical Control Division, Analytical Research Department, Merck & Co. Inc., Rahway, N. J.

penicillin has led to a variety of ultraviolet absorption methods for the quantitative estimation of this important antibiotic.

Herriott (4) was the first to publish an ultraviolet method for penicillin estimation. His method utilized the relatively high absorption at 322 mμ of a degradation product of penicillin as a rapid method for penicillin estimation. In this method, the absorption of the original solution is measured at 322 mμ; degradation of the penicillins present is effected by heating for 15 minutes at 100° in 0.3 *M* acetate buffer (*p*H 4.6) and the increase in absorption at this same wave length is measured. Although rapid, this technique does not differentiate between the various possible penicillins and is apparently useful only in determining the total quantity of penicillins present in solution over the range of 5 to 75 μg. with a reported accuracy of ±5%.

Philpotts, Thain and Twigg (5) suggested the use of the ultraviolet absorption of the phenyl group for the estimation of benzylpenicillin in the presence of other penicillins. The choice of a spectrographic technique, employing a wedge cell and photographic plate matching, is inconvenient for the analytical laboratory since the procedure is more cumbersome and time-consuming than the utilization of modern spectrophotometers designed for absorption studies.

Grenfell, Means and Brown (6) have recently described a convenient and rapid ultraviolet absorption method developed in the Charles Pfizer & Co., Inc., Laboratories, for the determination of benzylpenicillin. Their procedure involves:

(*a*) Use of specific rotation of the relatively pure penicillin solution to be tested as a measure of the *total* penicillins present.

(*b*) Measurement of the characteristic ultraviolet maxima of the benzyl group at 258 and 263 mμ to determine the benzylpenicillin present.

These authors recognize the possible interference of parahydroxy benzylpenicillin (penicillin X) and use Macpherson's (7) modification of the Pauly reaction to note the presence or absence of this penicillin in the sample studied. Their method involves the measurement of the optical density of the solution at 263 mμ and subtraction from this value of the optical density at 280 mμ. The latter is taken as a measure of degradation products and interfering substances which would increase the 263 absorption over and above that for the benzyl group present. This correction is found to be applicable, at the concentrations used, only when the density at 280 mμ is 0.10 or less; at higher values it is recommended that the sample be purified by conversion to the ammonium salt.

In his discussion on "A System of Penicillin Analysis" at the Chicago meeting of the American Chemical Society on April 21, 1948, G. B. Levy (8) reviewed in some detail a spectrophotometric technique developed in the Schenley Laboratories for estimation of benzylpenicillin. This method utilized the benzyl group absorption bands (in 95% ethanol solution) at 264.5 and 268.5 mμ. The difference in absorption between the peak at 264.5 mμ and the dip at 263 mμ is taken as a measure of the benzylpenicillin present. By measuring this difference the interference caused by extraneous absorbing material is minimized. Since a comparatively large temperature effect was found, constant temperature should be maintained in the absorption cell compartment.

In the Merck laboratories an ultraviolet method (9) employing more specific but limited corrections for possible common degradation products has been in routine use for about two years. The correction is based upon the thorough study of the ultraviolet absorption of the penicillins and their degradation products made by N. R. Trenner (10). These studies have indicated that the most common degradation products of penicillins are penicillenic acid and penamaldates. The E_m for penicillenic acid is 28,000 at its maximum at 320 mμ, and 885 at 257 mμ. The E_m for penamaldates is about 17,000 at its maximum at 280 mμ and 7500 at 257 mμ. Thus, it is possible by three precise absorption readings: (1) to measure at 257 mμ the sum of the absorption due to benzylpenicillin (at its maximum) together with the residual absorption due to the *usual* major degradation products, (2) to measure the absorption due to penicillenic acid at its maximum and thus calculate its effect on the benzyl peak at 257 mμ, and (3) to measure the absorption due to penamaldates at their absorption maximum and thus calculate their effect on the benzyl peak. Since the ratio of the absorptions for penicillenic acid at 257 mμ and at 320 mμ is 885:28,000, a factor of 1:30 is sufficiently precise for correction purposes. For penamaldates the ratio of 7500 to 17,000 approximates a 1:3 factor. In this method, then, the absorption of the solution of the relatively pure sample is measured at 257, 280, and 320 mμ. One-third of the measured density for the solution at 280 mμ and one-thirtieth of the measured density at 320 mμ are deducted from the measured density at 257 mμ. This results in a value closely approximating the absorption contributed by the benzyl group at 257 mμ. It should be borne in mind that penicilloates (10) present will be measured, of course, as penicillin.

In the above methods it is important to remember that the use of any ultraviolet absorption method is valid only in the absence of significant amounts of ultraviolet absorbing impurities. The benzylpenicillin content is measured indirectly through the benzyl absorption. Any aromatic impurity will have a large effect upon the precision of a benzylpenicillin assay by ultraviolet absorption technique. Such impurities are possible since substances like phenylacetic acid are frequently added to fermentation broths in order to increase the production of the benzyl form (11).

As far as other penicillins are concerned, the presence of parahydroxybenzylpenicillin (containing a phenol group) will definitely interfere with the determination of benzylpenicillin. Although other commonly occurring penicillins do not show the characteristic benzyl absorption peaks it should be pointed out that their ultraviolet absorption is appreciable at the lower wave lengths used (Fig. 1). Thus their presence, unless corrected by readings at other wave lengths, would tend to yield erroneously high values for benzylpenicillin. It is always advisable, in an assay of this type, to verify the actual presence of the benzyl peak by taking sufficient readings. Also, due to their microbiological activity, these other penicillins will disrupt correlation between ultraviolet and microbial assays.

In spite of these limitations, however, the ultraviolet absorption method for benzylpenicillin assay has been extremely useful in the production of high purity material because of its rapidity and reproducibility. Therefore, a comparative study of the various modifications suggested has been made in this laboratory. This study should be of interest to other laboratories because of the clinical importance of this antibiotic.

EXPERIMENTAL

A carefully tested standard sample of crystalline benzylpenicillin sodium was used to ascertain adherence to Beer's law. This sample had been in continual use as a standard in this laboratory and consequently many determinations had been performed on it by microbial means and by the official F. D. A. precipitation method. The average purity found for this sample by the F. D. A. N-ethyl piperidine method was 96.5 ± 0.3%. Low absorption at wave lengths above 270 mμ indicates a paucity of degradation products and a sensitive phenol test indicates the presence of less than 0.1% parahydroxybenzylpenicillin. An absorption curve of this sample is shown in Fig. 1.

1. Pfizer Method (Modified).—Six standard solutions of the above sample were prepared in the range 15 to 100 mg. benzylpenicillin per 100 ml. of solution, using distilled water at 25° as solvent. The absorption was immediately measured at 263 and 280 mμ. The arithmetical difference between these two values was then plotted against the concentration taken. The results are shown in Table I and Fig. 2.

The excellent straight line indicates rigid adherence to Beer's law under these conditions. The "unknowns" to be tested were then weighed out, dissolved in water, readings taken at 263 and 280 mμ, and the apparent benzylpenicillin content determined from this graph. The purities were calculated by dividing the benzylpencillin contents, as read from the graph, by the actual weights of samples taken. It should be noted that this constitutes a modification of the published method (6), which estimates the total penicillin by optical rotation. The use of

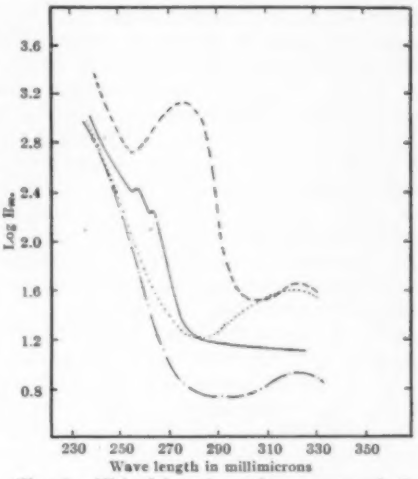


Fig. 1.—Ultraviolet absorption curves of the penicillins. Sodium Salt of: —— Penicillin G; - - - - Penicillin X; ----- Penicillin K; —·— Penicillin F.

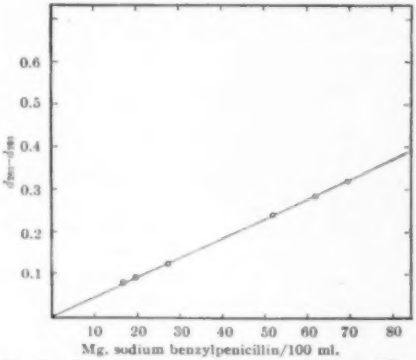


Fig. 2.—Calibration curve for the modified Pfizer method.

TABLE I.—DATA FOR STANDARD CURVES

Mg./100 Ml.	d_{257}	d_{263}	$d_{264.5}$	d_{280}	d_{320}
Aqueous					
16.8	0.139	0.091	...	0.010	0.006
19.9	0.148	0.105	...	0.012	0.009
27.5	0.209	0.136	...	0.010	0.010
51.7	0.395	0.262	...	0.018	0.013
61.8	0.472	0.309	...	0.023	0.020
69.5	0.520	0.339	...	0.019	0.018
Alcoholic					
15.0	...	0.064	0.069	...	...
20.1	...	0.085	0.094	...	...
25.0	...	0.105	0.117	...	...
31.2	...	0.131	0.142	...	...
33.5	...	0.141	0.159	...	...
34.9	...	0.140	0.159	...	...
43.0	...	0.181	0.199	...	...
44.8	...	0.187	0.212	...	...
48.7	...	0.202	0.228	...	...
51.1	...	0.211	0.237	...	...
67.7	...	0.287	0.312	...	...
89.7	...	0.377	0.421	...	...
139.9	...	0.589	0.652	...	...
190.0	...	0.786	0.876	...	...
230.4	...	0.958	1.061	...	...

a weighed sample avoids the inherent errors in carrying out rotation measurements and takes into account optically inert impurities.

2. Schenley Method (Modified).—Since alcoholic solutions are recommended in this method, additional portions of the standard sample were weighed out covering the range of 15 to 250 mg. per 100 ml., using 95% ethanol as solvent. The differences between the absorptions at 264.5 and 263 mμ were plotted against concentration taken. This is shown in Table I and Fig. 3. Here again adherence to Beer's law is indicated. The "unknowns" were then analyzed by dissolving weighed samples, measuring their absorption at the above wave lengths, and ascertaining, from the graph, the apparent benzylpenicillin content of the solution.

Since our laboratory deals primarily with highly purified samples, a simplification of the Schenley Method (8) is in order. Depending on the type of impurities present, the original method frequently indicates a negative intercept on the ΔE axis of the calibration curve. Our simplification assumes, in essence, that this intercept is zero. In samples assaying above 95% the error due to this simplification is less than 1%.

3. Merck Method.—In addition to the absorption values (263 and 280 mμ) measured on the samples for the "Pfizer Method," the absorption of these solutions was also measured at 257 and 320 mμ, as shown in Table I. One-third of the measured value at 280 mμ and one-thirtieth of the measured value at 320 mμ were deducted from the value at 257 mμ and the resultant value plotted against concentration. Figure 4 again indicates adherence to Beer's law. The "unknowns" were tested by reading the apparent benzylpenicillin content from the graph and comparing this value with the actual sample weight.

The precision of the three methods was ascertained by replicate determinations on a sample of benzylpenicillin which assayed 91.0% by the F. D. A. precipitation method. The results are summarized in Table II.

TABLE II.—PRECISION OF METHODS USED

Mg./100 ml.	Benzylpenicillin Content, %	Error, %
Schenley Method		
21.3	84.5	−6.5
55.2	97.4	+6.4
101.3	89.0	−2.0
102.5	91.5	+0.5
107.7	97.8	+6.8
116.6	88.5	−2.5
114.7	94.0	+3.0
130.2	89.0	−2.0
130.4	90.5	−0.5
149.5	94.5	+2.5
Pfizer Method		
18.8	93.0	+2.0
24.9	94.5	+3.5
36.3	93.5	+2.5
41.4	92.5	+1.5
47.7	92.5	+1.5
66.2	93.0	+2.0
69.4	91.5	+0.5
83.6	92.0	+1.0
Merck Method		
18.8	97.5	+6.5
24.9	98.0	+7.0
36.3	94.5	+3.5
41.4	94.5	+3.5
47.7	93.5	+2.5
66.2	94.5	+3.5
69.4	93.5	+2.5
83.6	93.5	+2.5

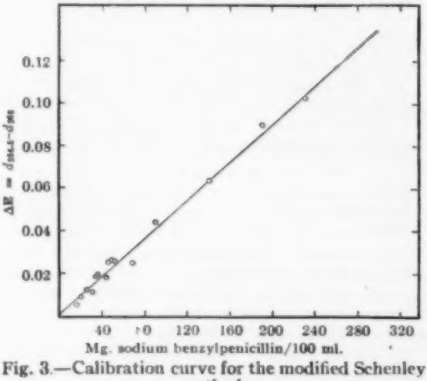


Fig. 3.—Calibration curve for the modified Schenley method.

The comparative accuracy of the three methods was determined by testing a group of samples that had been previously assayed by the official F. D. A. method. The results are given in Table III.

DISCUSSION

Statistical analysis of the above data (by J. H. Davidson of this Company) resulted in the following conclusions:

(a) A regression line for each of the calibration curves of the three methods gave *y* intercepts which were well within the error of determination of show-

TABLE III.—ACCURACY OF METHODS

Sample No.	F. D. A. Method, % G	Pfizer Method % G	Pfizer Method % Error	Schenley Method % G	Schenley Method % Error	Merck Method % G	Merck Method % Error
1	94.5	96.5	+2.0	90.0	−4.5	98.0	+3.5
2	94.5	95.0	+0.5	85.5	−9.0	96.5	+2.0
3	95.5	94.5	−1.0	...	...	97.5	+2.0
4	96.5	97.5	+1.0	89.0	−7.5	96.5	+0.0
5	96.0	97.5	+1.5	92.5	−3.5	98.0	+2.0
6	90.0	92.0	+2.0	89.0	−1.0	93.5	+3.5
7	94.5	95.0	+0.5	93.0	−1.5	94.0	−0.5
Av.	94.5	95.4	1.2	89.8	4.5	96.3	1.9

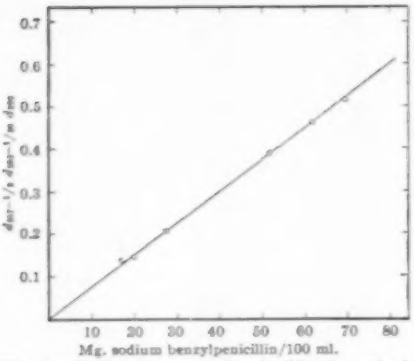


Fig. 4.—Calibration curve for the Merck method.

ing a zero density reading for zero concentration. This is in accordance with Beer's Law.

(*b*) The slopes of these calibration curves, which may be defined as the change in density function of the solution for each milligram increase in benzylpenicillin concentration (per 100 ml.) were found to be:

Pfizer	0.00460 ± 0.00006
Schenley	0.00045 ± 0.00003
Merck	0.00746 ± 0.00003

The ± figure is the range within which the slope is known with a probability of 0.99. Of the three methods the Merck procedure gives the largest density change per unit concentration, and the smallest relative error in precision.

(*c*) Using the data from Table III, the average difference between each method and the official F. D. A. chemical method, together with the expected range (probability of 0.99) within which the difference should fall, is given in Table IV.

CONCLUSIONS

Of the four methods suggested in the literature for ultraviolet assay of benzylpenicillin salts, the photographic has not been included for manipulatory reasons. Of the three spectrophotometric methods the one described by Grenfell, *et al.* (6) gives the best agreement with the official F. D. A. precipitation method. For routine controls the Merck method is also satisfactory. Two factors militate against the Schenley method. The necessary careful temperature control does not add to the attractiveness of this method; also, this method utilizes small differences between two relatively high optical density measurements. As may be seen from Fig. 3 an optical density difference of 0.005 unit represents 5% in the final assay. Although the Beckman spectrophotometer is a good instrument, its reproducibility is no better than 0.005 unit at the densities measured.

TABLE IV

Method	Average Difference from F. D. A., %	Difference Range
Pfizer	0.9	− 1.8 to +3.7
Schenley	−4.5	−12.8 to +3.8
Merck	1.8	− 2.2 to +5.8

SUMMARY

A comparative study has been made of three spectrophotometric methods currently in use for assay of benzylpenicillin.

REFERENCES

(1) *Federal Register*, **12**, 2222(1947).
(2) Mader, W. J., and Buck, R. R., *Anal. Chem.*, **20**, 284 (1948).
(3) Sheehan, J. C., Mader, W. J., and Cram, D. J., *J. Am. Chem. Soc.*, **68**, 2407(1946).
(4) Herriott, R. M., *J. Biol. Chem.*, **164**, 725(1946).
(5) Philpotts, A. R., Thain, W., and Twigg, G. H., *Nature*, **159**, 839(1947).
(6) Grenfell, T. C., Means, J. A., and Brown, E. V., *J. Biol. Chem.*, **170**, 527(1947).
(7) Macpherson, H. T., *Biochem. J.*, **36**, 59(1942).
(8) Levy, G. B., Shaw, D., Parkinson, E. S., and Fergus, D., *Anal. Chem.*, **20**, 1159(1948).
(9) Colon, A. A., and Frediani, H. A., *Bol. Col. Quim. Puerto Rico*, **4**, 20(1947).
(10) Chemistry of Penicillin, National Academy of Science Monograph, Princeton University Press, forthcoming publication.
(11) Coghill, *et al.*, U. S. Patent No. 2,423,873.

A Chromatographic Study of Pyrethrum Flowers*,†

By G. H. HAMOR, J. F. SUCHY, and C. H. WALDON

This work consists of an investigation of the adsorption of extracts of Chrysanthemum (*Pyrethrum*) *Cinerariaefolium* on a chromatographic column. The study was undertaken to determine the possibility of the separation of a pyrethrum extract mixture on a chromatographic column into different colored bands, with the subsequent identification, estimation, and isolation of the component parts (especially pyrethrins and cinerins) of the extract.

SINCE THE introduction of pyrethrum as an insecticide in the middle of the nineteenth century, an intensive investigation has been undertaken to determine its active principles and to find an accurate method of quantitative analysis. Recent work (1) seems to indicate that the toxicity is due to four compounds instead of the two pyrethrins previously considered the only active principles. These four constituents are the two esters of chrysanthemum monocarboxylic acid with the cyclic ketonic alcohols, pyrethrolone and cinerolone; and the two methyl esters of chrysanthemum dicarboxylic acid with these alcohols.

Because the present chemical methods of determining pyrethrin content are relatively long and complicated with many chances for error, this study of adsorption analysis of pyrethrum extracts was undertaken to determine the possibility of resolving and quantitatively separating the pyrethrins by means of chromatography.

EXPERIMENTAL

The material used consisted of flowers of *Chrysanthemum cinerariaefolium* grown in the drug garden of the School of Pharmacy, Montana State University. Using the National Formulary method of analysis (2) the flowers averaged 0.81% Pyrethrin I and 0.58% of Pyrethrin II.

Alumina was first tried as an adsorbent. Several organic solvents were used to extract the pyrethrum flowers. A petroleum ether extract gave an orange-red band which could not be developed or eluted.

Powdered sucrose, Lloyd's Reagent, Celite, calcium oxide, calcium hydroxide, calcium carbonate, bentonite, Permutit and magnesium carbonate were tested as adsorbing agents and were found to be unsatisfactory. Heavy magnesium oxide of the ordinary commercial grade gave the most well-defined bands. The extract used was prepared by subjecting a 492.5-Gm. sample of *Chrysanthemum cinerariaefolium* to twenty hours of continuous extraction in a Friedericks Extractor with petroleum ether as a solvent and evaporating the resulting solution to exactly 200 cc. This extract assayed 0.007360 Gm. of Pyrethrin I per cc. and 0.006614 Gm. of Pyrethrin II per cc. Thirty cubic centimeters of this solution was adsorbed on a magnesium oxide column 25 mm. in diameter. Three zones were observed: a top band, greenish yellow in color; a middle zone, orange-yellow in color; and a colorless bottom band.

Various solvents were tested in the development of the chromatogram, none being highly successful. In most cases a pink-colored layer was observed but this band could never be distinguished in the extruded column. Different eluents were tried but elution could be obtained only by subjecting the dry powder to twenty hours of continuous extraction with petroleum ether. On analysis of the different zones, approximately 65% of the pyrethrins were found to be in the middle orange-yellow layer, with 20% in the top greenish yellow band, and a negligible amount in the colorless band. Pyrethrins I and II were present in approximately the same percentage. Eighty-five per cent recovery from the column was obtained.

In an effort to attain further concentration, the orange-yellow band containing the bulk of the pyrethrins was extracted and run through another chromatographic column. No distinctive layers were obtained, the entire column assuming a homogeneous bright yellow color.

In order to rule out the possibility of other plant principles interfering with the adsorption of the pyrethrins, it was necessary to obtain the pyrethrins in a purified form. Starting with a soluble pyrethrum oleoresin,[1] and using the method of Barthel, Haller, and La Forge (3), a concentrate containing 90% of total pyrethrins was prepared.

Of this concentrate, 1.5 Gm. was diluted to 25 cc. with petroleum ether. Two cubic centimeters of this solution was run through a column of heavy magnesium oxide. A distinct pink upper layer 2 mm. in width was formed and, on standing or developing, a very light red lower band 50 mm. in width was seen. Differentiation of the layers could not be attained. Elution was obtained only by dissolving the dry powder with 10% hydrochloric acid. The pyrethrins were then extracted by shaking with petroleum ether. On analysis, 29% of the pyrethrins were found in the pink layer and 69% in the lower red layer, thus effecting a total recovery of 98%.

* Received Sept. 2, 1948, from the School of Pharmacy, Montana State University, Missoula, Mont.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.

† This paper is based on a thesis presented to the Graduate School of Montana State University by Glenn H. Hamor in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

[1] S. B. Penick Co., 50 Church St., New York 7, N. Y.

SUMMARY AND CONCLUSIONS

When a pyrethrum extract was chromatographed, the bulk of the pyrethrins was concentrated in a highly colored orange-yellow zone which could not be differentiated.

When the extract was freed of most of the interfering plant principles and run through a column, two differently colored zones, one dark pink and the other light pink in color, were observed. Both of these zones contained pyrethrins.

An effective resolution of pyrethrins could not be obtained by chromatography, possibly due to the fact that the pyrethrins, being esters, were decomposed by the alkalinity of the magnesium oxide. This hydrolysis would not be detected on analysis because the assay for pyrethrins is based on the reaction with acids freed from the esters.

REFERENCES

(1) La Forge, F. B., and Barthel, W. F., *J. Org. Chem.*, 10 114(1945).
(2) "The National Formulary," ed. 8, Mack Printing Co., Easton, Pa., 1946, p. 423.
(3) Barthel, W. F., Haller, H. L., and La Forge, F. B. *Soap*, 20, 121(1944).

Modified Assay Methods for Crude Drugs Involving the Removal of Interfering Substances by Enzymatic Digestion. II. Modified Assay Method for Belladonna Root*

By HENRY LOUIS VERHULST,† LOUIS ARRIGONI,‡ and LOUIS FISCHER§

The N. F. method for the determination of the alkaloid content was modified by digesting the drug with a mixture of "Taka-Diastase" and pancreatin. After digestion, the mixture was extracted with chloroform and the official method was carried out to completion. Satisfactory results were obtained with a material improvement in the procedure from the standpoint of decreasing the time, equipment involved, and the facility of operation.

THE OFFICIAL ASSAY of belladonna root requires maceration of the drug overnight followed by Soxhlet extraction for three hours, or an alternate percolation method. In either process the alkaloids are separated and purified by the immiscible solvent procedure. The alkaloids are then determined volumetrically using 0.02 *N* acid and alkali. The main objections to the official assay are: (*a*) the length of time required; (*b*) the tendency to form emulsions during the shaking-out process; and (*c*) the extraction of relatively large quantities of impurities along with the alkaloids.

This study was undertaken to develop a process which would eliminate the use of Soxhlet equipment, overnight maceration, some of the emulsions, impurities from the extractions, and still yield satisfactory results. The method of approach chosen to remedy these objections and to simplify the official assay was to hydrolyze enzymatically the proteins and carbohydrates, and in this manner attempt to eliminate the extraction of interfering substances and impurities.

EXPERIMENTAL

Before undertaking the investigation of a new process using enzymes,[1] it was first necessary to have information regarding the composition of the drug; consequently, a partial phytochemical analysis of the sample was made.

The drug, No. 40 powder belladonna root, was thoroughly mixed and placed in a tightly closed

* Received Aug. 30, 1948, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Washington, Seattle, Wash.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
† Former Associate Chemist, State Laboratory, University of Washington, Seattle, Wash.
‡ Assistant Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Washington, Seattle, Wash.
§ Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Washington, Seattle, Wash.

[1] Subsequent to the presentation of this paper for publication the following reference, "Utilisation of Diastase in the Extraction of Alkaloids from Crude Drugs," by H. N. Gupta and S. B. Sen Gupta appeared in *Chemical Abstracts*, 42, 6059b (Aug. 20, 1948). This work involved a similar study on the use of enzymatic degradation of cellular material for the purification of the alkaloids in several plant drugs. Priority is claimed for this type of investigation by virtue of the master's thesis by A. J. Anderson, entitled "A Study of Assay Methods for Colchicum Corm and Seed Including the Removal of Interfering Substances by Enzymatic Digestion," which was accepted by the University of Washington Library, June, 1946.

container. All determinations using official methods were made in triplicate (1), or until satisfactory results were obtained. The values, calculated on a moisture-free basis, are reported in Table I.

TABLE I.—PARTIAL PROXIMATE COMPOSITION BELLADONNA ROOT

	%
Moisture	6.43
Starch	38.45
Reducing Sugars	2.35
Sucrose	12.20
Ash	11.70
Protein	6.73
Petroleum Ether Extract	0.47
Pentosans	None

For comparative purposes, the alkaloidal content of the drug was determined by the N. F. VIII procedure (2) and the results are presented in Table II.

TABLE II.—RESULTS OF N. F. ASSAY OF BELLADONNA ROOT

Sample	%
1	0.33
2	0.32
3	0.35
4	0.35
5	0.35
6	0.34
7	0.35
Av.	0.34

Assay of Belladonna Root by Enzymatic Digestion with Pancreatin and Taka-Diastase.—The selection of suitable enzymes to hydrolyze the starch and proteins in belladonna root was limited to pancreatin and "Taka-Diastase." Gelatinizing the starch in the drug by heating in a boiling water bath before subjecting it to the action of pancreatin and subsequent extraction of the active constituents gave low results, probably because of hydrolysis of the alkaloids. In an effort to overcome the decrease in alkaloidal yield, the procedure was modified by eliminating the preliminary heating. The large amount of starch present indicated that Taka-Diastase could be used to advantage as the Taka-Diastase digests about 300 times its weight of starch while pancreatin digests 25 times its weight of the carbohydrate. The use of the pancreatin-Taka-Diastase mixture required a shorter digestion time than pancreatin alone.

In the preliminary treatment of the samples with enzymes, three different methods were used. First, a 10-Gm. sample was mixed with 20 cc. of *p*H 6.7 sodium phosphate buffer and 30 cc. of water. The mixture was then placed in a beaker of boiling water for twenty minutes to gelatinize the starch. After cooling, 1.5 Gm. of pancreatin was added and the mixture digested at 55° for two and a half hours, at which time a drop of iodine T. S. gave no blue color when added to one drop of the homogeneous mixture. The second method was similar to the above except that the sample was not heated. The third procedure was identical with the latter save that 0.5 Gm. of Taka-Diastase was added in addition to the pancreatin and the digestion time was reduced to two hours.

After complete digestion in each instance, the sample was transferred quantitatively to a separatory funnel. Several organic solvents were used to determine which was the best for the extraction of the alkaloids. Chloroform showed the least tendency to emulsify and consequently was adopted for the procedure which follows.

Fifty cubic centimeters of chloroform was added to the separatory funnel; the solution was neutralized with ammonium hydroxide; and an excess of 2 cc. was added. The alkaloid was then extracted with the organic solvent and the chloroform layer drawn off and passed through a dry filter into a second separatory funnel. The alkaloid was extracted with three more 25-cc. portions of chloroform. Two cubic centimeters of the final chloroform extraction was evaporated to dryness, the residue was taken up in several cubic centimeters of 0.1 *N* sulfuric acid and tested with Valser's reagent. The fourth extraction always gave a negative test for alkaloids. The combined chloroform extractions were then evaporated to about 50 cc. and extracted with three 25-cc. portions of 0.5 *N* sulfuric acid. The combined sulfuric acid extractions were neutralized and shaken out with chloroform. The total chloroform extractions were evaporated on a water bath to dryness and the flask heated for fifteen minutes. The residue was redissolved in a small volume of the solvent, evaporated to dryness on a water bath, and again heated for fifteen minutes. This treatment was repeated for a third time. The alkaloids in the residue were determined as directed in the N. F. VIII. The results obtained from the extraction after the pancreatin-Taka-Diastase digestion were comparable with those obtained by the N. F. method as may be observed in Table III.

Effect of Enzymes on Loss of Atropine.—In reviewing the literature, it was noted that loss of atropine by hydrolysis in the presence of natural enzymes was reported (3). To determine if any such loss of alkaloid occurred during the digestions with Taka-Diastase and pancreatin, a known quantity of atropine was added to three 10-Gm. samples of Belladonna root and analyzed.

To insure that the atropine used was pure, 200 mg. was made up to 200 cc. in a volumetric flask with a known quantity of 0.02 *N* sulfuric acid and a 50-cc. aliquot was titrated using standard alkali. This determination in duplicate showed each 50-cc. portion contained 50.1 mg. of atropine. Ten cubic centimeters of the remaining solution was added to each of two samples and 20 cc. to a third sample. The samples were then analyzed for atropine by the procedure described above.

The results, as shown in Table IV, indicate there was no loss of alkaloid during the enzymatic digestion.

SUMMARY

The work was divided into two parts: (*a*) partial phytochemical analysis of the drug and (*b*) the study of enzymatic digestion of the root followed by the extraction of the alkaloids from the digested mixture.

TABLE III.—COMPARISONS OF RESULTS OBTAINED BY N. F. AND PROPOSED METHODS

Sample	N. F. Method, %	Pancreatin Modification with Heat, %	Pancreatin Modification without Heat, %	Pancreatin-Taka-Diastase Modification, %
1	0.33	0.29	0.35	0.36
2	0.32	0.30	0.34	0.35
3	0.35	0.30	0.35	0.35
4	0.35	..	..	0.36
5	0.35	..	..	0.35
6	0.34	..	..	0.35
7	0.35	..	..	..
Av.	0.34	..	..	0.35

TABLE IV.—COMPARISON OF ATROPINE RECOVERED FROM SAMPLE WITH KNOWN QUANTITY ADDED

Weight of Sample, Gm.	Alkaloidal Content of Sample, Mg.	Amount of Atropine Added to Sample, Mg.	Total Alkaloid Extracted, Mg.	Amount of Added Atropine Recovered, Mg.
10.6442	36.2	10	46.4	10.2
10.2657	34.9	10	44.7	9.8
10.7330	36.5	20	56.7	20.2

The official method for the determination of the alkaloid content was modified by digesting the drug with a mixture of Taka-Diastase and pancreatin. After digestion, the mixture was extracted with chloroform and the official method was carried out to completion. By adding a known quantity of atropine to a sample of the drug and carrying out the modified procedure, it was shown that no loss of alkaloid occurred during the digestion.

Results comparable to those of the official method may be obtained with a material improvement in the procedure from the standpoint of decreasing the time and equipment involved and the facility of operation.

REFERENCES

(1) "Methods of Analysis," Association of Official Agricultural Chemists, ed. 6, Washington, D. C., 1945.
(2) "National Formulary," ed. 8, Mack Printing Company, Easton, Pa., 1946.
(3) Todd, J. P., *J. Roy. Tech. Coll.* (Glasgow), **2**, 353 (1930).

The Antibacterial Properties of Cyanine*

By DONALD W. McKINSTRY†

The bacteriostatic effect of cyanine (quinoline blue) on a variety of pathogenic microorganisms *in vitro* was determined. The data obtained indicate that this compound is remarkably effective against *Corynebacterium diphtheriae* and the Gram-positive cocci. The amount of drug tolerated by Swiss albino mice compared favorably with that of several other medicaments employed today in therapy of certain infectious diseases. The therapeutic possibilities of this compound in human and veterinary medicine are discussed.

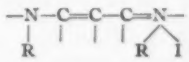
THE MARKED antibacterial action of cyanine (1,1′-di-isoamyl-4,4′-quinocyanine iodide, $C_{29}H_{35}N_2I$) was noted during a recent survey of synthetic organic dyes for antibacteriophage activity (1). Although the compound was found to be devoid of any significant antiviral properties, its extraordinary bacteriostatic effect against *Staphylococcus aureus* prompted an investigation of its activity in this respect with other pathogenic microorganisms.

The first biologic property of cyanine was discovered by Paul Ehrlich (2) in 1887 when he succeeded in staining central nervous tissue *in vivo* with an alcoholic solution of the dye mixed with serum. Behring (3), two years later, found that the dye was a good bactericide but did not investigate its selective antibacterial properties. Tagami (4) confirmed Behring's discovery and also noted that its bactericidal action was not influenced by the *p*H of the medium. Tappeiner

* Received July 6, 1948, from the Department of Bacteriology, The Pennsylvania State College, State College, Pa.
† Present address: Laboratory of Infectious Diseases, Microbiological Institute, National Institute of Health, Bethesda, Md.

and Jodlbauer (5) discovered that the compound was endowed with protozoacidal properties and Martinotti (6) found it to be very toxic for tadpoles.

Browning and his co-workers (7) in 1926 began an intensive systematic study of the antiseptic action of the quinoline dyes in an effort to determine the relationship between chemical structure and bactericidal properties. They found that the structure,



present in cyanine and acriflavine, in which a trivalent, basic nitrogen atom is joined to a pentavalent nitrogen atom by a carbon chain in which there are alternating double bonds, appeared to be essential for bactericidal action. *Staphylococcus aureus* and *Escherichia coli* were employed as test organisms in this study. Although the results obtained by these workers amply confirmed the previously published reports concerning the remarkable bactericidal action of cyanine, the compound was not investigated with regard to its application as a therapeutic agent in microbiological diseases of man and domesticated animals. During the course of this investigation Brooker and Sweet (8) reported the effect of a series of cyanine, styryl and related dyes on certain bacteria, parasites, protozoa and viruses. One of the compounds studied—(1-amyl- 2,5 - dimethyl - 3 - pyrrole)(1,6 - dimethyl - 2 - quinoline) di-methinecyanine chloride—inhibited the growth of *Lactobacillus casei*, *L. arabinosus*, *Staphylococcus fecalis*, and *E. coli in vitro*. None of the compounds was therapeutically effective against a number of infectious agents *in vivo*. Cyanine is employed primarily today for the isochromatization of photographic plates and to a limited extent by histologists for staining fats, bone tissue, and lignified membranes.

This paper describes the bacteriostatic effect of cyanine on a variety of pathogenic microorganisms *in vitro* and its toxicity for experimental animals.

MATERIALS AND METHODS

Test Microorganisms.—A variety of pathogenic microorganisms, including Gram-positive cocci, Gram-negative cocci, Gram-positive spore-forming rods, and Gram-negative nonspore-forming rods, were employed in this study in an effort to determine the microbiological limits of the antibacterial action of the drug.

Media.—A standard medium of a constitution known to satisfy the nutritional requirements for optimal growth and activity was used for each microorganism.

Inoculum.—In order to make the assay as rigorous and critical as possible, a massive inoculum consisting of 0.1 ml. of a vigorous eighteen- to forty-eight-hour broth culture was used to inoculate each tube of drug-treated broth as well as the culture control.

Antibacterial Assay.—Serial dilutions of the drug were prepared in the medium of choice for the organism under investigation. Each tube of drug-treated medium was then inoculated as described above. Adequate culture controls and drug controls were included in each assay. The cultures were incubated for from forty-eight to ninety-six hours at the temperature optimal for growth of the organism in question. All culture tubes were carefully examined and the extent of bacterial growth determined under standard conditions at the end of the incubation period.

Criterion of Antibacterial Potency.—The degree of inhibition of growth as evidenced by the concentration of bacterial cells in the drug-treated medium as compared with that of the untreated bacterial control was used as the criterion of antibacterial potency. The concentration of bacterial cells in each culture was determined by turbidimetric methods using the transillumination technique with the aid of a comparator box and a standard light source. The bacteriostatic end point was considered to be the highest dilution of drug that completely inhibited growth of the microorganism studied.

Acute Toxicity for Mice.—In an effort to determine whether the toxic effects of the compound were too great to warrant exploration of its possibilities as a therapeutic agent for infectious diseases, the acute toxicity for mice was determined. Graded doses of the drug in physiologic saline were administered intraperitoneally to young Swiss albino mice. Ten mice were employed in each group for a given dosage. The animals were observed for a period of seven days and examined daily for evidence of toxic manifestations. The LD_{50} dose of drug for this experimental animal was determined by the method of Reed and Muench (9).

EXPERIMENTAL RESULTS

The bacteriostatic effect of cyanine against the microorganisms employed in this study is summarized in Table I.

The toxicity of the drug in terms of the LD_{50} dose for young Swiss albino mice is presented in Table II.

DISCUSSION

Examination of the bacteriostatic assay data presented in Table I reveals that cyanine is remarkably effective *in vitro* against a variety of pathogenic microorganisms. Its deleterious effect in minute concentration on *Corynebacterium diphtheriae* and the pathogenic Gram-positive cocci is especially noteworthy. The extraordinary susceptibility of *S. aureus* to this drug coupled with its strong affinity for osseous tissue indicates that its possible therapeutic application in staphylococcal osteomyelitis merits investigation.

The amount of cyanine tolerated by the mouse (Table II) compares favorably with that of several

TABLE I.—ANTI-BACTERIAL ACTION OF CYANINE

Microorganism[a]	Medium	Growth of Microorganism in Indicated Dilution of Drug												Culture Control	Bacteriostatic Titer
		10^{1}	10^{2}	10^{3}	10^{4}	10^{5}	10^{6}	10^{7}	10^{8}	10^{9}	10^{10}	10^{11}	10^{12}		
Staph. aureus (PJ1B)	Nutrient broth	–	–	–	–	–	–	–	–	–	*	++	++++	++++	10^{9}
Strep hemolyticus (C-203)	Veal infusion broth	–	–	–	–	–	–	*	+	+++	++++	++++	++++	++++	10^{5}
Strep. agalactae	Veal infusion broth	–	–	–	–	–	*	+	++	+++	++++	++++	++++	++++	10^{5}
Strep. dysgalactae	Veal infusion broth	–	–	–	–	–	*	+	+++	++++	++++	++++	++++	++++	10^{5}
Strep. uberis	Veal infusion broth	–	–	–	–	–	–	+	+	++	++++	++++	++++	++++	10^{6}
Diplococcus pneumoniae	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^{5}
Neisseria intracellularis	Veal infusion Destrose broth	–	–	–	–	–	–	–	*	+	+	+++	++++	++++	10^{7}
Eberth. typhosa (P-63)	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^{5}
Sal. paratyphi	Veal infusion broth	–	–	–	–	–	–	+++	++++	++++	++++	++++	++++	++++	10^{6}
Sal. schott-müelleri	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^{5}
Sal. enteritidis	Veal infusion broth	–	–	–	–	–	*	++	++++	++++	++++	++++	++++	++++	10^{5}
Sal. gallinarum	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^{5}
Sal. typhimurium	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^{5}
Shig dysenteriae (K-7C)	Veal infusion broth	–	–	–	–	–	*	+++	++++	++++	++++	++++	++++	++++	10^{6}
Shig. dysenteriae (Sonnei)	Veal infusion broth	–	–	–	–	–	+++	++++	++++	++++	++++	++++	++++	++++	10^{5}
Coryne. diphtheriae	Veal infusion ascitic fluid medium	–	–	–	–	–	–	–	*	++	++++	++++	++++	++++	10^{7}
Klebsiella. pneumoniae	Veal infusion broth	–	+	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	10^{1}
Pseudomonas aeruginosa	Nutrient broth	–	–	–	–	+	++	++++	++++	++++	++++	++++	++++	++++	10^{4}
Clostridium. welchii	Brewer's medium	–	–	–	–	–	+++	++++	++++	++++	++++	++++	++++	++++	10^{6}

[a] The results obtained with *Neisseria gonorrheae* and *mycobacterium tuberculosis* (human strain) were inconclusive.

TABLE II.—ACUTE TOXICITY OF CYANINE FOR MICE

Dilution of Compound	Inoculum, Ml.	Dose, Mg.	Route Adm.	Number of Mice	Weight of Mice, Gm.	—Death of Mice (Days)— 1	2	3	4	5	6	7	Mortality, %	LD_{50} Mg./Kg.
10^2	0.1	1	IP[a]	10	20	9	1	0	0	0	0	0	100	
10^3	0.1	0.1	IP	10	20	0	0	0	0	0	0	0	0	
10^4	0.1	0.01	IP	10	20	0	0	0	0	0	0	0	0	16
10^5	0.1	0.001	IP	10	20	0	0	0	0	0	0	0	0	

[a] IP, intraperitoneally.

other medicaments employed today in the chemotherapy of certain infectious diseases. The history of chemotherapy is prominently marked by the discouraging cases where compounds discovered to be singularly effective against a particular infectious agent were later found to be too toxic for the host when tested against the infectious process *in vivo*. Fortunately, the limited toxicity of cyanine does not preclude exploration of its therapeutic possibilities in human and veterinary medicine.

Further studies of the effect of this compound in experimental bacterial infections is planned and will be reported in a later publication.

SUMMARY AND CONCLUSIONS

1. The bacteriostatic effect of cyanine (quinoline blue) on a variety of pathogenic microorganisms is described.

2. The toxicity of the drug in terms of the LD_{50} dose for young Swiss albino mice is given.

3. The possible application of the compound in the therapy of certain microbiological diseases is discussed.

REFERENCES

(1) McKinstry, D. W., Conn, E. F., and Farrell, M. A., in press.
(2) Ehrlich, Paul, *Therap. Monatsh.*, **88**(1887).
(3) Behring, O. Von, *Z. Hyg. Infektionskrankh.*, **7**, 173 (1889).
(4) Tagami, M., *Tôhoku J. Exptl. Med.*, **18**, 482(1932).
(5) Tappeiner, H. Von, and Jodlbauer, A., *Deut. Arch. klin. Medi.*, **80**, 427(1904).
(6) Martinotti, G., *Z. wiss. Mikroskop.*, **5**, 305(1888).
(7) Browning, C. H., Cohen, J. B., Ellingworth, S., and Gulbranson, R., *J. Path. Bact.*, **29**, 317(1926).
(8) Brooker, L. G., and Sweet, L. A., *Science*, **105**, 670 (1947).
(9) Reed, L. J., and Muench, H., *Am. J. Hyg.*, **27**, 493 (1938).

Experiments on the Constitution of Umbellatine*

By R. CHATTERJEE†

Based on the experimental work described in this report, the correct formula for umbellatine is believed to be $C_{21}H_{23}O_4N$. The alkaloid has been found to contain one methylenedioxy, two methoxyl and four hydroxyl groups. A structural formula (VII) is suggested for umbellatine

THE ALKALOID UMBELLATINE, $C_{21}H_{23}O_4N$, has been isolated from several Indian species of *Berberis* (1) and *Mahonia* (2). It has been used with success in the treatment of oriental sore. The pharmacological action of umbellatine is similar to that of berberine but more intense, except in cardiovascular responses which are less than those caused by berberine (3).

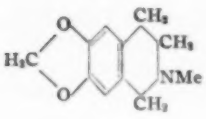
It has been shown that umbellatine is a tertiary base. It contains one methylenedioxy, two methoxyl, and four hydroxyl groups (4). On heating with zinc dust in a current of hydrogen, *iso*quinoline is obtained, which shows that the nitrogen atom together with its methyl group is attached to a ring and that it belongs definitely to the *iso*quinoline group of alkaloids. From a comparison of the ultraviolet absorption curve of umbellatine with that of berberine, it has been concluded that 2:3-methylenedioxytetrahydro*iso*quinoline ring forms a part of the umbellatine nulceus (4). Moreover, to degrade umbellatine into a nitrogen-free compound, Hofmann degradation has to be repeated twice for the nitrogen valences are involved in a hydrogenated ring system.

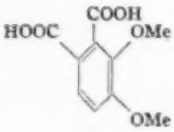
The Linking of the Benzene Nucleus to the Isoquinoline Nucleus.—Umbellatine gives *ortho*hemipinic acid on oxidation with potassium permanganate (5) and opianic acid with manganese dioxide and sulfuric acid. In both cases of oxidation, nitrogenous fragment of the

* Received June 15, 1948, from the Department of Chemistry, University College of Science and Technology, Calcutta 9, India.
† The author expresses his thanks to the University of Calcutta for a Sir P. C. RAy Research Fellowship.

umbellatine molecule cannot be isolated. The *iso*quinoline part of the molecule may be weaker due to the presence of substituents like phenolic hydroxyl groups.

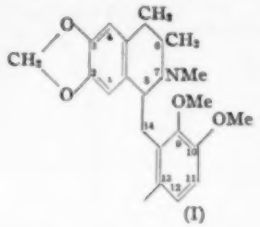
The evidence for the presence of a methylenedioxytetrahydro*iso*quinoline skeleton (4) and a hemipinic or opianic acid nucleus gives a fair indication to the structure of umbellatine.





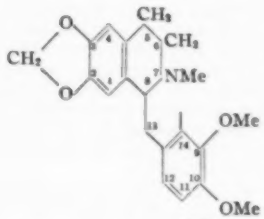
If the linking of the dimethoxybenzene portion to the tetrahydro*iso*quinoline nucleus is assumed to be of benzyl*iso*quinoline type, where the dimethoxybenzene portion is linked to the *iso*quinoline part by a methylene group, the easily breakable point will be at the methylene group producing a carboxyl or an aldehyde group. For instance, papaverine produced on alkali fusion, veratric acid (6), and laudanosine, on oxidation with manganese dioxide and sulfuric acid, yielded veratraldehyde (7). Here the production of opianic acid from umbellatine indicates a benzyl*iso*quinoline structure with a substituent in the *ortho* position to the linkage, which can be oxidized to an aldehyde group. Another suggestion would be the hydrastine structure, for both hydrastine (8) and narcotine (9) yielded opianic acid on oxidation. Since umbellatine has not been found to undergo hydrolytic decomposition, the lactonic group is, in all probability, absent from it. The corydaline structure (10) is not applicable to it, for a methyl group is already attached to the nitrogen atom in it. The nitrogen atom common to two rings, with a group attached to it, has to be pentavalent and, as such, the formation of salts of umbellatine of the type B, HX with acids cannot be explained. A base like berberine or palmatine (10) with a hydroxyl group attached to the nitrogen atom, where the latter is pentavalent, only forms salts by the elimination of water. The skeleton of umbellatine is represented by (I) or (II), the benzyltetrahydro*iso*quinoline structure with a substituent in position 13 or 14 which can be oxidized to a carboxyl or an aldehyde group.

Discussion of the Position of the Hydroxyl Groups.—The positions 2 and 3 are occupied by the methylenedioxy group; 9 and 10 by the two methoxyl groups. Four of the positions 1, 4, 5, 6, 8, 13, and 14 are occupied by the four hydroxyl groups. The aldehyde group of the opianic acid is developed in position 13



4 OH groups

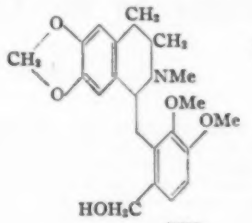
(I)



4 OH groups

(II)

of formula (I), after fission of the linkage at 14. It is very likely obtained by the oxidation of a primary alcoholic group placed at that position in the base. The structure of umbellatine is, therefore, preferably represented by the skeletal formula (I), with a primary alcoholic group definitely in position 13, for the location of this group in 14 in formula (II) cannot explain the production of

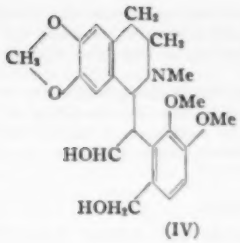


3 OH groups

(III)

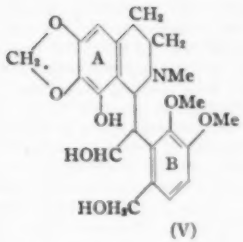
opianic acid. The skeletal formula of umbellatine can be written as (III). In hydrastine and narcotine the linking at 14 is by a latent :CHOH group. Considering a similar possibility of a potential hydroxyl group in 14 (II), the structural formula of umbellatine can be extended further to (IV). One of the two remaining hydroxyl groups is positively phenolic in character. Either of the positions 1 and 4 can only be allotted to it, when umbellatine will be represented either by (V) or (VI).

There remains now to locate the position of only one hydroxyl group, probably one of the two hydroxyl groups, which can be acetylated.



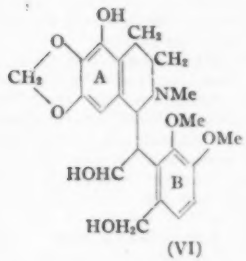
2 OH groups

(IV)



1 OH group

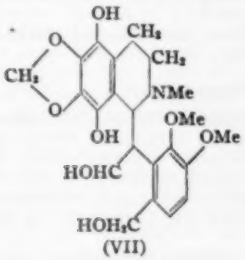
(V)



1 OH group

(VI)

The phenolic character of this hydroxyl group can only be inferred from a slight hyperchromic effect in the absorption spectra and the *p*H value (5.7) of its 0.5 per cent aqueous solution. Unfortunately in cases of oxidation by potassium permanganate, manganese dioxide and sulfuric acid, chromic acid and sulfuric acid, chromic acid and acetic acid, the nitrogenous fragment or a product containing the methylenedioxy group cannot be isolated. This shows that the ring A containing the methylenedioxy group is not as stable to oxidation as ring B, containing the two methoxyl groups. The instability may be due to the presence of phenolic hydroxyl group or groups in ring A. Methyl umbellatine is oxidized by different agents, but neither a product containing ring A nor a nitrogenous fragment can be isolated. Had there been only one phenolic group in position 1 or 4, and if this group were methylated before oxidation, a fragment like cotarnic acid would be very likely to be obtained. So the instability of the ring A in both cases of umbellatine and its methyl derivative may well be due to the phenolic hydroxyl groups both in positions 1 and 4. Positions 5 and 6 are not considered because alkaloids have not been isolated in which these positions carry a substituent (11). A hydroxyl group cannot possibly be placed at position 8, since two hydroxyl groups are not very likely present in adjacent positions, and no alkaloid of benzyltetrahydro-*iso*quinoline structure is yet known with hydroxyl group at 8. The structural formula of umbellatine is finally suggested to be (VII).



(VII)

EXPERIMENTAL

Zinc Dust Distillation.—Umbellatine (2 Gm.) was mixed with zinc dust (20 Gm.) and heated strongly in a combustion tube over a free flame, the air in the tube being replaced by a current of hydrogen. The reaction mass was kept stirred by constant rotation of the tube, which was heated to redness within ten minutes. The reaction product which was a brownish liquid was taken in dilute hydrochloric acid (5%) and the mixture was slightly warmed. The residue in the tube was cooled and then digested three times with ether (50 cc. each time). The brown ether solution was shaken with the above hydrochloric acid solution. The ether layer was treated separately. The ether was removed, and the residue, a tarry product, could not be successfully worked up. The acid solution was then made alkaline with dilute sodium hydroxide solution (5%) and the mixture was repeatedly extracted with ether. The ether layer was washed with water and then dried over anhydrous sodium sulfate. The residue obtained, after removal of ether, was subjected to a high vacuum distillation, when a drop of a colorless liquid was obtained at 70°/0.3 mm. pressure. The liquid gradually became yellow in air. It was identified as *iso*quinoline by the preparation of the following derivatives.

Isoquinoline Picrate.—A part of the liquid was taken in dry ether and mixed with a solution of picric acid in ether. It was kept for an hour in a refrigerator until a yellow-colored picrate separated. The crude picrate melted at 195°. The picrate was difficultly soluble in alcohol from which it was repeatedly crystallized. The pure product was obtained as pale yellow needles which vaporized to a certain extent at 200° and finally melted sharply at 215°. The picrate of a synthetic specimen of *iso*quinoline melted similarly at 215° after partial

vaporization at 200°. The m. p. did not change when the two samples were mixed together.

Anal.—Calcd. for $C_9H_7N \cdot C_6H_3N_3O_7$: N, 15.3. Found: 15.5.

Isoquinoline Chloroplatinate.—The liquid obtained by high vacuum distillation was mixed with dilute hydrochloric acid and treated with a solution of chloroplatinic acid (5%) when a pale orange precipitate separated. On warming the mixture, the precipitate went in solution, from which pale orange-colored crystals separated on cooling. The chloroplatinate which was recrystallized from water shrank at 258° and melted with decomposition at 260–262°. An authentic specimen of *iso*-quinoline chloroplatinate had the same m. p. and when both samples were mixed, the m. p. was not depressed.

Anal.—Calcd. for $(C_9H_7N)_2H_2PtCl_6$: Pt, 28.2. Found: 27.9.

Hofmann Degradation

Iodomethylate of Methyl Umbellatine.—Methyl umbellatine was dissolved in methyl alcohol and treated with excess of methyl iodide. On leaving overnight brilliant yellow crystals of the methiodide were obtained which decomposed without melting.

Anal.—Calcd. for $C_{23}H_{25}O_9NI$: I, 22. Found: 19.8.

Methine of Methyl Umbellatine.—Iodomethylate of methyl umbellatine (4.0 Gm.) was placed in boiling water (200 cc.) and refluxed with potassium hydroxide (45 Gm.) in methyl alcohol (25 cc.) for six hours, when most of the substance decomposed and the methine was obtained as a thick, viscous brown mass. The brown mass was boiled for some time to get rid of the methyl alcohol, and then extracted with ether. The ether extract could not be solidified.

Iodomethylate of the Methine.—The crude methine was dissolved in methyl alcohol and then treated with excess of methyl iodide. The methiodide was crystallized from methyl alcohol as yellow needles, which decomposed above 200°.

Anal.—Calcd. for $C_{22}H_{26}O_9NI$: C, 72.7; H, 7.9; I, 33.4. Found: C, 72.0; H, 8.1; I, 33.0.

Decomposition of the Iodomethylate of the Methine.—When an alcoholic solution of the iodomethylate was refluxed with potassium hydroxide solution for eight hours, trimethylamine was obtained. The vinyl derivative thus obtained forms a heavy oil, which was taken up in chloroform. The extract after removal of chloroform could not be solidified.

Oxidation of the Iodomethylate of the Methine.—The iodomethylate (1.0 Gm.) in water (100 cc.) was treated with potassium permanganate solution (1%) at the ordinary temperature, until the color of the solution remained pink. The mixture was then warmed on a water bath, to complete the oxidation, which required further addition of the permanganate solution until pink. The filtered solution was decolorized by sulfur dioxide and then concentrated *in vacuo* to about 100 cc. A large amount of inorganic matter was filtered off. The solution was then acidified with hydrochloric acid and continuously extracted with ether. The ether was evaporated and the pasty mass of the residue was distilled *in vacuo*, when colorless needles were obtained at 150–160°/0.1 mm. The needles on crystallization from water were eventually identified to be hemipinic acid, obtained previously by the permanganate oxidation of the base umbellatine itself.

Oxidation of the Vinyl Compound.—The vinyl compound obtained by the decomposition of the iodomethylate of the methine was oxidized by the method described above, to obtain finally crystals of hemipinic acid.

Oxidation Experiments

Manganese Dioxide and Sulfuric Acid.—Umbellatine (2.5 Gm.) was dissolved in a mixture of sulfuric acid (5 Gm.) and water (200 cc.) and mechanically stirred with manganese dioxide (2 Gm.) for an hour on a water bath. The mixture slowly became brown and turbid. The reaction was rather lazy and the heating was continued for another two hours. After cooling the mixture was filtered. The brown-colored granular residue was dried in air and extracted with ether in a Soxhlet apparatus. On removal of ether no residue was left behind. The filtrate was extracted exhaustively with ether. The ether was removed and the residue was sublimed as a white crystalline substance at 160°/0.1 mm. The aqueous solution was then made ammoniacal and exhausted with ether. The residue obtained, on removal of ether, was sticky and could not be worked up successfully.

Identification of Opianic Acid.—The white crystalline substance, thus obtained was crystallized from water as fine colorless prisms; m. p. 147°. It did not depress the m. p. of opianic acid obtained by the oxidation of narcotine by concentrated sulfuric acid and manganese dioxide (12). The opianic acid obtained from narcotine melted at 150°.

Anal.—Calcd. for $C_{10}H_{10}O_5$: C, 57.1; H, 4.8. Found: C, 57.1; H, 4.6.

Oxime of Opianic Acid.—Opianic acid (1 mol.) was dissolved in a dilute solution of potassium hydroxide (4 mols.), mixed with hydroxylamine hydrochloride (1.5 mols.), and left overnight. The mixture was then acidified with hydrochloric acid and allowed to stand, when needle-shaped crystals separated which were collected and crystallized twice from warm water; m. p. 82°.

Anal.—Calcd. for $C_{10}H_{11}NO_5$: N, 6.2. Found: N, 6.1.

Potassium Permanganate.—Umbellatine hydrochloride (5 Gm.) was dissolved in warm water (150 cc.) and treated with potassium carbonate (1.0 Gm.) solution and then oxidized by slowly running in a solution of potassium permanganate (5%). The decolorization of the permanganate was very rapid at the start, and as soon as about 330 cc. of the solution was added decolorization slowed down appreciably and continued warming was necessary to remove the last traces of the pink color of the permanganate solution.

The solution was left overnight and then saturated with carbon dioxide gas and filtered from the manganese precipitate. The filtrate and the washings were evaporated completely to dryness on a water bath, and the residue was soxhletted with absolute alcohol. The residue obtained after the removal of the last traces of alcohol was dissolved in water and the brown aqueous solution was treated with diluted sulfuric acid when a small quantity of sticky matter separated which could not be solidified. The sticky

matter was removed by filtration and the filtrate was extracted continuously with ether (100 cc. in four installments) by shaking each time in a mechanical shaker for eight hours. The ethereal solution on removal of ether yielded a pale yellow oily residue which solidified when kept in a vacuum desiccator over concentrated sulfuric acid for a week. It was then sublimed at 0.1 mm. pressure when a minute quantity of a yellow liquid separated first at 140–150°, and then a transparent crystalline solid, moist with the liquid at 150–160°. The moist solid when dried on a porous plate melted at 125–157°.

The moist solid was well washed with small quantities of ether, dried and powdered and warmed with concentrated hydrochloric acid and kept overnight and filtered from the acid solution and repeatedly washed with cold water in small amounts. The filtrate and the washings yielded a very small quantity of a semisolid which could not be successfully handled. The residue was then dried; m. p. 155–160°. The m. p. further rose to 177–178.5° on slow crystallization of the solid from boiling water.

Anal.—Calcd. for $C_{19}H_{18}O_8$: C, 53.1; H, 4.43. Found: C, 52.96; H, 4.75.

Hemipinic Acid.—To identify the hemipinic acid thus obtained, the m. p. of the sample was compared with that of hemipinic acid prepared from opianic acid, which in turn was obtained from narcotine (13). The hemipinic acid as obtained from narcotine melted at 179–180° and, when mixed with that obtained from umbellatine, melted at 179°.

Ethylimide of Hemipinic Acid.—The ethylimide derivative of hemipinic acid was prepared according to Liebermann (14) by dissolving the purified acid obtained from umbellatine in an aqueous solution of ethylamine (30%) and evaporating it to dryness. The residue thus obtained was heated carefully over a naked flame, and then exhausted with boiling methyl alcohol. On cooling the ethylimide separated as needles; m. p. 90°. The m. p. did not change even on high vacuum sublimation of the ethylimide at 125–135°/0.15 mm. pressure. The ethylimide of hemipinic acid, obtained from narcotine, was purified by the author by high vacuum sublimation and by crystallization from water, and was found to melt sharply at 92°. This m. p. was not depressed by the ethylimide of hemipinic acid obtained from umbellatine. This shows that the hemipinic acid thus obtained is 3,4 dimethoxy phthalic acid.

Anal.—Calcd. for $C_{12}H_{13}O_4N$: N, 5.9. Found: N, 5.63.

Chromic Acid and Sulfuric Acid.—The base (2 Gm.) was dissolved in water (100 cc.) and warmed on a water bath with a mixture of chromic acid (1.0 Gm.) and concentrated sulfuric acid (1.4 Gm.) and water (20 cc.). After one hour an equal quantity and, after another half hour, half the quantity of chromic acid were added. After two hours when no more chromic acid was being used up, the excess was reduced by sulfur dioxide. The mixture was filtered and extracted exhaustively with ether.

Treatment of the Ether Layer.—On removal of ether, a small quantity of sticky substance remained which, on vacuum sublimation, yielded colorless needles at 130°/0.08 mm. The needles, on crystallization from boiling water, melted at 177°, and eventually were identified to be hemipinic acid, through the ethylimide of the acid.

Treatment of the Aqueous Layer.—The green-colored aqueous solution was nearly neutralized by barium hydroxide solution. The precipitate was filtered and boiled with water. The remaining sulfuric acid was removed from the combined filtrate by barium carbonate. The neutral solution thus obtained, was evaporated, and the residue extracted with alcohol. The residue left, on removal of alcohol, was dissolved in water, and heated carefully with ammonium hydroxide; no solid separated. The ammoniacal solution was then extracted with water. The ether extract was distilled in high vacuum, and neither a solid nor a liquid separated.

The Molecular Formula of Umbellatine.—The molecular formula of umbellatine was reported to be $C_{21}H_{21}O_8N$, on the basis of combustion analyses of the base and its salts. Now a study of its degradation products has led to changing the formula to $C_{21}H_{23}O_8N$, without any appreciable change in its percentage composition. Found: C, 59.0, 59.2; H, 5.33, 5.4; N, 3.3, 3.3; OMe, 14.4, 14.4. $C_{21}H_{23}O_8N$, $^1/_2H_2O$ requires C, 59.2; H, 5.6; N, 3.3; OMe, 14.6. $C_{21}H_{21}O_8N$, $^1/_2H_2O$ requires C, 59.4; H, 5.2; N, 3.3; OMe, 14.6%. Pt, 15.68, 15.71.

Anal.—Calcd. for $(C_{21}H_{23}O_8N)_2$, H_2PtCl_6; Pt, 15.73. Found: Pt, 15.72%.

SUMMARY

1. Umbellatine is an alkaloid obtained from some of the Indian species of *Berberis* and *Mahonia*. Its formula is correctly represented by $C_{21}H_{23}O_8N$.

2. Umbellatine is a tertiary base, and it contains one methylenedioxy, two methoxyl, and four hydroxyl groups.

3. On oxidation with manganese dioxide and sulfuric acid, it produces opianic acid; with potassium permanganate or chromic acid and sulfuric acid, *ortho*hemipinic acid is obtained.

4. On zinc dust distillation, it gives *iso*quinoline.

5. The structural formula of umbellatine is suggested to be (VII).

REFERENCES

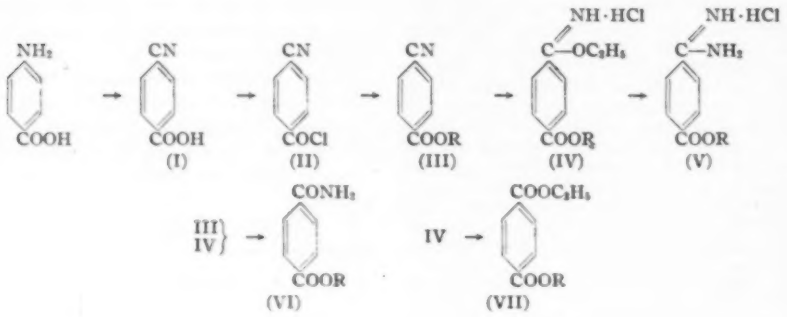
(1) Chatterjee, R., *J. Indian Chem. Soc.*, **17**, 289(1940); THIS JOURNAL, **30**, 247(1941); *Science and Culture*, **7**, 619 (1942).
(2) Chatterjee, R., THIS JOURNAL, **33**, 210(1944).
(3) Gupta, J., and Kahali, B. S., *Indian J. Med. Research*, **32**, 53(1944).
(4) Chatterjee, R., *J. Indian Chem. Soc.*, **19**, 233(1942).
(5) Chatterjee, R., *ibid.*, **19**, 385(1942).
(6) Goldschmiedt, G., *et al.*, *Monatsh.*, **24**, 681(1903).
(7) Pyman, J., *J. Chem. Soc.*, **95**, 1266(1909).
(8) Freund, M., and Will, W., *Ber.*, **20**, 88(1887).
(9) Beckett, G. H., and Wright, C. R. A., *J. Chem. Soc.* **28**, 573(1875).
(10) Henry, T. A., "The Plant Alkaloids," ed. 3, P. Blakiston's Son, Philadelphia, 1939.
(11) Manske, R. H. F., *Canadian J. Research*, **B17**, 51 (1939).
(12) Wöhler, F., *Ann.*, **50**, 1(1844).
(13) Beilstein, F., "Handbuch der Organischen Chemie," Auflage III, Band II, ed. 1896, 1994.
(14) Liebermann, C., *Ber.*, **19**, 2275(1886).

The Synthesis of Some Esters of *p*-Carboxybenzamidine*

By FRANK E. DI GANGI and OLE GISVOLD†

An attempt was made to synthesize some esters of *p*-carboxybenzamidine wherein their structure resembled that of the common local anesthetics in general use. The compounds synthesized differ from the esters of *p*-aminobenzoic acid only in that the amidino group replaces the *p*-amino group. The methyl through hexyl series of esters of *p*-carboxybenzamidine were prepared.

THE OBJECT of this investigation was to prepare some esters of *p*-carboxybenzamidine wherein their structure resembled that of the common local anesthetics of the *p*-aminobenzoic acid type. The compounds synthesized differ from the esters of *p*-aminobenzoic acid only in that the amidino group replaces the *p*-amino group. The study was undertaken to try to improve the general method for the preparation of unsubstituted amidines, particularly those containing an ester grouping in the molecule. The chemical properties of the imido ester hydrochloride (1–4), an intermediate, led to the preparation of some mixed esters of terephthalic acid and some esters of terephthalamic acid.



R = H, CH_3, C_2H_5, C_3H_7, *n*-C_4H_9, *n*-C_5H_{11}, or *n*-C_6H_{13}.

EXPERIMENTAL

p-Cyanobenzoic Acid (I).—An alkaline solution of cuprous cyanide was prepared from the cuprous chloride, which was obtained from 0.6 *M* of copper sulfate, 0.625 *M* of sodium chloride, 0.3 *M* of sodium bisulfite, and 0.51 *M* of sodium hydroxide, and 1.25 *M* of sodium cyanide in 200 cc. of water to which was added 0.5 *M* of monohydrated sodium carbonate. To this solution was added a diazonium solution prepared from 1 *M* of hydrochloric acid, 0.5 *M* of *p*-aminobenzoic acid, and 0.5 *M* of sodium nitrite. The diazonium solution was maintained at 0–5°, while it was slowly added to the alkaline cuprous cyanide solution with stirring. The resulting mixture was heated at 60° for thirty minutes to drive off the nitrogen and the carbon dioxide that formed. The alkaline reaction mixture was cooled in a refrigerator for several days to allow a large mass of crystalline sodium *p*-cyanobenzoate to form. This crystalline mass represents about 50% of the total yield obtained. The mother liquor obtained after filtration was acidified with hydrochloric acid and filtered to collect the precipitated crude *p*-cyanobenzoic acid which was then treated with ammonium hydroxide to dissolve out the *p*-cyanobenzoic acid as the ammonium salt. The insoluble inorganic copper salts were filtered off. The filtrate was acidified and chilled and filtered to obtain an impure *p*-cyanobenzoic acid. The impure product was purified after several recrystallizations from hot water and decolorized with charcoal. The sodium salt of the acid obtained previously was dissolved in water, the solution was acidified, and the precipitated acid recrystallized from water. This modification in the method of preparation of the acid produced a yield of 68%. Melting point found 219°, reported 219° (5).

p-Cyanobenzoyl Chloride (II).—To one mole of *p*-cyanobenzoic acid in a one-liter, round-bottom flask equipped with a reflux condenser and a calcium chloride drying tube, was added 294 cc. of thionyl

* Received August 11, 1948, from the College of Pharmacy, University of Minnesota, Minneapolis, Minn.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
Abstracted in part from a thesis submitted by Frank E. Di Gangi to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.

chloride. The mixture was heated on a steam bath for one hour. The excess thionyl chloride was then removed with heat under vacuum of a water pump. The residual oil was poured over crushed ice. The crystalline acid chloride was filtered off, dried, and recrystallized from petroleum ether. (When smaller reaction quantities are used the excess thionyl chloride may be allowed to evaporate off spontaneously in a well-ventilated hood and the crude acid chloride then recrystallized from petroleum ether.) The total yield was 90%. The melting point found 65°, reported 65° (6).

p-Cyanobenzoic Acid Esters (III).—To 0.2 *M* of *p*-cyanobenzoyl chloride was added 30 cc. of the appropriate alcohol in small portions. The mixture was allowed to stand for a short time when an evolution of heat occurred and gaseous hydrogen chloride formed. Water and sodium bicarbonate were added to the reaction mixture and then the neutralized mixture was chilled and filtered. The precipitated ester was then recrystallized from alcohol and water.

β-Diethylaminoethyl Ester of p-Cyanobenzoic Acid Hydrochloride.—To 0.2 *M* of *p*-cyanobenzoyl chloride in 150 cc. of benzene was added slowly a solution of 0.188 *M* of β-diethylaminoethyl alcohol in 100 cc. of benzene. The reaction mixture was heated for one hour under a reflux condenser over a steam bath. The crude ester which had separated was collected on a filter and dried. It was purified by recrystallization from absolute alcohol. The mother liquor was diluted with ether for a further yield of product. The total yield was 84.7%. Melting point was 159–160°.

Anal.—Calcd. for $C_{14}H_{19}O_2N_2Cl$: C, 59.46; H, 6.77. Found: C, 59.47; H, 6.99.

p-Carbethoxyimidobenzoic Acid Ester Hydrochlorides (IV).—One-tenth mole of the *p*-cyanobenzoic acid ester, 100 cc. of anhydrous ether, and 10 cc. of absolute alcohol were introduced into a 250-cc. flask equipped with a two-hole rubber stopper for a gas inlet tube and a calcium chloride drying tube and the mixture was weighed and chilled to 0°. Anhydrous hydrogen chloride was passed into the solution at a moderate rate. After thirty minutes the gas inflow was stopped and the flask weighed. Between 21 and 27 Gm. of gas was absorbed in this

TABLE I.—ESTERS OF *p*-CYANOBENZOIC ACID

Ester	Yield, %	Melting Point, °C. Found	Melting Point, °C. Reported	Formula	Analysis, % C and H Found	Analysis, % C and H Calcd.
Methyl	75	62	62	$C_9H_7O_2N$	.....	.....
Ethyl	85.8	54	54	$C_{10}H_9O_2N$	.....	.....
Propyl	68.8	40–41	..	$C_{11}H_{11}O_2N$	C, 69.90 H, 6.01	C, 69.82 H, 5.86
n-Butyl	77	56–57	..	$C_{12}H_{13}O_2N$	C, 70.91 H, 6.51	C, 70.92 H, 6.45
n-Amyl[a]	63.5	18–19	..	$C_{13}H_{15}O_2N$	C, 71.59 H, 7.09	C, 71.86 H, 6.96
n-Hexyl	63	45–46	..	$C_{14}H_{17}O_2N$	C, 72.93 H, 7.74	C, 72.69 H, 7.41

[a] The amyl ester was an oil at room temperature and was purified by distillation under vacuum.

TABLE II.—*p*-CARBETHOXYIMIDOBENZOIC ACID ESTER HYDROCHLORIDES

Ester	Yield, %	Melting Point, °C. Found	Melting Point, °C. Reported	As Free Base[a] Melting Point, °C. Found	As Free Base[a] Melting Point, °C. Reported
Methyl	77	202–203	.....	Oil	...
Ethyl	70	173–174	178–179	73–74	Oil
Propyl	68	114–115	.....	Oil	...
n-Butyl	81	74–76	.....	Oil	...
n-Amyl	70	92–94	.....	Oil	...
n-Hexyl	70	101–102	.....	Oil	...

[a] These were prepared by direct neutralization with a 5% sodium bicarbonate solution and extracted with ether. The oils were reported as such at room temperature.

TABLE III.—ESTERS OF *p*-CARBOXYBENZAMIDINE HYDROCHLORIDE

Ester	Yield, % Method 1	Yield, % Method 2	Melting Point, °C.	Empirical Formula	Analysis % C and H Found	Analysis % C and H Calc.
Methyl	78.5[b]	85.2[g]	326–327 Over 250 (S)	$C_9H_{11}O_2N_2Cl$	C, 50.83 H, 5.40	C, 50.37 H, 5.16
Ethyl	89[c]	84[h]	207–208 found 217–218 rep.	$C_{10}H_{12}O_2N_2Cl$	C, 52.67 H, 5.83	C, 52.52 H, 5.73
Propyl	63[d]	75[i]	170–171.5	$C_{11}H_{13}O_2N_2Cl$	C, 54.77 H, 6.52	C, 54.43 H, 6.23
n-Butyl	84[e]	75[j]	149–150	$C_{12}H_{17}O_2N_2Cl$	C, 56.49 H, 7.23	C, 56.12 H, 6.76
n-Amyl	70[f]	89[k]	141–142	$C_{12}H_{19}O_2N_2Cl$	C, 57.68 H, 7.14	C, 57.66 H, 7.07
n-Hexyl	87[e,m]	85[l]	139–141	$C_{14}H_{21}O_2N_2Cl$	C, 59.63 H, 7.84	C, 59.04 H, 7.46

[a] Ammonium sulfate replaced ammonium chloride since the ammonium chloride reaction produced the amidine hydrochloride which was low melting and had water of hydration.

[b–m] The amounts of imido ester hydrochlorides reacted were as follows: *b*, 8.3 Gm.; *g*, 4.0 Gm.; *c*, 9.75 Gm.; *h*, 10 Gm.; *d*, 7.0 Gm.; *i*, 2.3 Gm.; *e*, 10 Gm.; *j*, 7.0 Gm.; *f*, 10 Gm.; *k*, 4.6 Gm.; *m*, 5.0 Gm.; *l*, 3.0 Gm.

time. The flask was stoppered and set in the refrigerator for several to ten days. During this time the imido ester hydrochloride crystals were filtered off and the mother liquor returned to the refrigerator for additional yields. The exhausted mother liquor was then treated to obtain the unreacted ester by removing the excess gas and evaporating off the ether and recrystallizing the ester from alcohol and water.

p-Carboxybenzamidine Esters (V).—The initial steps in *Method 1* (7, 8) were the neutralization of the ester of *p*-carbethoxyimidobenzoic acid hydrochloride with a 5% solution of sodium bicarbonate (9) and the extraction with ether in a separatory funnel. The ether extracts were combined and dried over anhydrous sodium sulfate. The ether was then evaporated off and the oily base weighed. The base was then dissolved in 30 cc. of alcohol and the exact molal quantity of ammonium chloride was dissolved in 8 cc. of water and added to the alcoholic solution of the base. This mixture was heated at 60–70° for three to four hours or until the mixture was neutral or slightly acid to nitrazine pH indicator paper. The reaction mixture was then concentrated and cooled. Acetone and ether were added until the mixture became turbid but no liquid layers separated. The reaction mixture was chilled in the refrigerator and fine needles of the amidine hydrochloride crystallized out.

The crystals were contaminated with unreacted ammonium chloride and they contained water of hydration. In order to remove the ammonium chloride and water of hydration, the product was converted to the free amidine base with a 10% solution of sodium hydroxide and extracted several times with chloroform. The chloroformic extracts were then combined and dried over anhydrous sodium sulfate. The chloroform solution was concentrated to a small volume and petroleum ether added to precipitate the free amidine base. The base was purified by recrystallization from benzene and petroleum ether. An ether solution of hydrogen chloride was added to an ether solution of the base which then yielded a crystalline precipitate of the amidine hydrochloride.

In *Method 2*, the ester of *p*-carbethoxyimidobenzoic acid hydrochloride was dissolved in 30 cc. of alcohol. Ammonium hydroxide was then added dropwise until a faint odor of ammonia persisted. The mixture was then heated at 60–70° for two hours, after which it was concentrated and cooled. After cooling, acetone and ether were added to the mixture until the solution became turbid. On chilling, long needles of the amidine hydrochloride formed and were filtered off. The amidine hydrochlorides were purified in the same manner as outlined under Method 1.

Table IV gives the melting points of the free bases, picrates and sulfates of the esters of *p*-carboxybenzamidine:

p-Carboxybenzamidine Ester Carbonates.—By virtue of the strong basic character of the free amidines, they readily formed the carbonates in crystalline form when an aqueous solution of the amidine hydrochloride was neutralized with excess sodium bicarbonate solution. They were dried in a vacuum desiccator over phosphorous pentoxide and recrystallized from absolute alcohol.

Terephthalic Acid Esters (VI).—The *p*-carbethoxyamidobenzoic acid ester hydrochlorides readily dissolved in water. On standing a turbidity developed which, in some cases, turned into a crystalline precipitate and, in others, settled out as an oil at 25°. The separated products were mixed or symmetrical terephthalic acid esters.

Terephthalamic Acid Esters (VII).—The *p*-cyanobenzoic acid esters were covered with 78%

TABLE IV.—AMIDINES AND THEIR DERIVATIVES

Ester	Amidine Base, M. p., ° C.	Amidine Picrate,[a] M. P., ° C.	Amidine Sulfate,[b] M. P., ° C.
Methyl	137–139	231–232	241–242 (decomp.)
Ethyl	110–112	213–214	252
Propyl	92–94	204–205	209–210
n-Butyl	63–64	192–193	199–200
n-Amyl	84–85	202–203	172–174
n-Hexyl	Oil (at 25)	195–196	210–211[c]

[a] Prepared in the usual manner from the free base.
[b] Prepared from the free base by neutralization.
[c] Prepared also from the ammonium sulfate reaction.

TABLE V.—AMIDINE CARBONATES

Ester	Melting Point, ° C.	Empirical Formula	Analysis, % C and H— Found	Calc.
Methyl	143–144	$C_{19}H_{22}O_7N_4$	C, 54.58 H, 5.65	C, 54.51 H, 5.30
Ethyl	108–109	$C_{21}H_{26}O_7N_4$	.....	.....
Propyl	102–103	$C_{23}H_{30}O_7N_4$	C, 57.77 H, 6.59	C, 58.21 H, 6.37
n-Butyl	86–88	$C_{25}H_{34}O_7N_4$	C, 59.49 H, 7.07	C, 59.74 H, 6.82
n-Amyl	91–92	$C_{27}H_{38}O_7N_4$	C, 60.73 H, 7.52	C, 61.11 H, 7.22
n-Hexyl	93–94	$C_{29}H_{42}O_7N_4$	.....	.....

TABLE VI.—TEREPHTHALIC ACID ESTERS

Ester	Melting Point, ° C.	Ester	Melting Point
Mono-ethyl	171 found 171 rep.	Propyl-ethyl	Oil
Methyl-ethyl[a]	35–36	*n*-Amyl-ethyl	Oil
Diethyl	44 found 44 rep.	*n*-Hexyl-ethyl	Oil

[a] This compound was analyzed for $C_{11}H_{12}O_4$; calcd. C, 63.45, H, 5.81; found C, 63.63, H, 5.97.

TABLE VII.—TEREPHTHALAMIC ACID AND ESTERS

Compound	Melting Point, °C.	Empirical Formula	—Analysis, % C and H— Found	Calc.
Terephthalamic Acid	Over 300 S at 250 rep. Over 360 found	$C_8H_7O_3N$	.....	.....
Methyl Ester	201 rep. 201 found	$C_9H_9O_3N$	.....	.....
Ethyl Ester[a]	171–172	$C_{10}H_{11}O_3N$	C, 61.98 H, 5.86	C, 62.16 H, 5.68
Propyl Ester[b]	131–132	$C_{11}H_{13}O_3N$	C, 63.72 H, 6.46	C, 63.75 H, 6.32
n-Butyl Ester[c]	131–133	$C_{12}H_{15}O_3N$	C, 64.93 H, 6.98	C, 64.84 H, 6.80
n-Amyl Ester	123–124	$C_{13}H_{17}O_3N$	C, 66.92 H, 7.53	C, 66.36 H, 7.32
n-Hexyl Ester	126–127	$C_{14}H_{19}O_3N$	C, 67.85 H, 7.88	C, 67.44 H, 7.68

[a] Formed also by hydrolysis of *p*-carbethoxyimidobenzamide hydrochloride.
[b, c] Formed also from the imido ester hydrochlorides by heating them under vacuum at their melting points.

sulfuric acid and allowed to stand for forty-eight hours. At the end of this time the mixture was poured on crushed ice and the amide crystallized out. The crude product was filtered off and washed with water and then purified by several recrystallizations from water. These compounds were obtained in yields of 60% to 65%.

***p*-Carbethoxyimidobenzoic Acid Hydrochloride.**—Five grams of *p*-cyanobenzoic acid was suspended in 100 cc. of anhydrous ether and 25 cc. of absolute alcohol in a 250-cc. flask and chilled to 0°. Gaseous hydrogen chloride was passed in for one hour at a moderate rate. The reaction mixture was set aside in the refrigerator for several days at the end of which time the mixture was filtered and the residue washed with ether. The yield of product was 90% The melting point was over 300°, with decomposition and sublimation. In one attempt, only absolute alcohol was used as the solvent and the product isolated was the ethyl ester of *p*-carbethoxyimidobenzoic acid hydrochloride which formed through esterification of the free carboxyl group.

***p*-Carboxybenzamidine Hydrochloride.** — One gram of *p*-carbethoxyimidobenzoic acid hydrochloride was dissolved in 20 cc. of a 10% solution of ammonia in absolute alcohol. After a few minutes the solution became turbid. After standing in sealed bottles for four days at room temperature, the precipitate was filtered off and the filtrate heated to remove the excess ammonia and to concentrate the liquid to small volume. The solution was then cooled and acidified with hydrochloric acid and the precipitate filtered off. Five-tenths of a gram of product was obtained by recrystallization from alcohol that melted at 283°.

Anal.: Calcd. for $C_8H_5O_2N_2Cl.H_2O$: C, 43.94; H, 5.07. Found: C, 44.37; H, 5.26.

***p*-Cyanobenzamide.**—Five grams of *p*-cyanobenzoyl chloride was added in small portions to 20 cc. of ammonium hydroxide. The reaction was vigorous. Upon completion of the reaction, the mixture was cooled and filtered and washed with water. The insoluble residue, which was the amide when recrystallized from water, was obtained in the form of long slender needles. The yield was 2.9 Gm. of a product that melted at 224° which corresponded with that reported in the literature.

***p*-Carbethoxyimidobenzamide Hydrochloride.**—Three grams of *p*-cyanobenzamide were dissolved in 100 cc. of absolute alcohol and the solution chilled to 0°. Gaseous hydrogen chloride was passed into the solution for thirty minutes. Twenty grams were absorbed. The solution was placed in the refrigerator for two days; then the crystals which formed were filtered off and washed with ether. The yield was 2.6 Gm. (71%). It melted above 300°.

Terephthalamamidine Hydrochloride.—*p*-Carbethoxyimidobenzamide hydrochloride (2.5 Gm.) was dissolved in alcohol and warmed while ammonium hydroxide was added in the manner described under Method 2. The mixture was heated for several hours at 60–70° at the end of which time the solution was concentrated. Ether was added and the amidine hydrochloride precipitated out. One gram of product that melted at 284–285° was obtained by recrystallization from alcohol.

Anal.: Calcd. for $C_8H_{10}ON_2Cl$: C, 48.12; H, 5.09. Found: C, 47.66; H, 4.96.

SUMMARY

Six new amidines, containing ester groupings in their structure, were prepared, together with some of their derivatives.

Five new esters of *p*-cyanobenzoic acid were prepared in good yields.

Five new esters of terephthalamic acid were prepared from the corresponding nitriles and the imido ester hydrochlorides.

Mixed esters of terephthalic acid were prepared by hydrolysis of the corresponding imido ester hydrochlorides. The mono-ethyl, methyl-ethyl, and diethyl are solids, the propyl through hexyl-ethyl esters were obtained as oils.

The method of Pinner as modified by Knorr and Barber for the preparation of unsubstituted amidine salts has been applied to nitriles containing ester groups in converting them to amidines in high percentage yields. It was found that the water used to dissolve the ammonium chloride in this method was unnecessary.

An improved method for the preparation of amidines from the imido ester hydrochlorides was devised. The yields are comparable to or greater than the previous method. The conversion of the imido ester hydrochloride to the base and subsequent extraction with ether preparatory to reaction with ammonium chloride in a hydro-alcoholic solution is entirely eliminated by the direct addition of ammonium hydroxide to the imido ester hydrochloride in alcohol.

REFERENCES

(1) Pinner, A., "Die Imidoaether und ihre Derivate," Robert Oppenheim (Gustav Schmidt) Berlin, 1892.
(2) Derby, I. H., *Am. Chem. Jour.*, **39**, 437(1908).
(3) Chapman, A. W., *J. Chem. Soc.*, **1922**, 1676.
(4) Pinner, A., *Ber.*, **28**, 473(1895).
(5) Valby, E. P., and Lucas, H. J., *J. Am. Chem. Soc.*, **51**, 2718 (1929).
(6) King, H., *J. Chem. Soc.*, **1939**, 254.
(7) Knorr, A., *Ber.*, **50**, 229(1917).
(8) Barber, H. S., and Self, A. D. H., U. S. Patent 2,375,-611.
(9) Glickman, S. A., and Cope, A. C., *J. Am. Chem. Soc.*, **67**, 1012(1945).

The Spectrophotometric Determination of the Dissociation Constants of Theophylline, Theobromine, and Caffeine*

By ARTHUR TURNER, Jr.,† and ARTHUR OSOL‡

The results of a study of the ultraviolet spectrophotometric absorption characteristics of aqueous solutions of theophylline, theobromine, and caffeine at different *p*H values are reported. From these data the acidic dissociation constants of theophylline and theobromine, expressed as pK_a values, were found to be 8.6 and 10, respectively. The basic dissociation constants of each of the three substances correspond to a pK_b value of not less than 13 in any case.

THEOPHYLLINE (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), and caffeine (1,3,7-trimethylxanthine) all behave as weak bases. Theophylline and theobromine, each having an —NH— group in its molecule, can also function as acids; caffeine, not having such a group, cannot react as an acid. The possible mechanisms of acid behavior of theophylline and theobromine have been discussed by Ogston (1), who also measured their acidic dissociation constants potentiometrically.

A study of the ultraviolet spectrophotometric absorption characteristics of the three xanthine derivatives at different *p*H values appeared to be of interest, especially from the standpoint of the possibility of determining the acidic and basic dissociation constants of theophylline and theobromine, and the basic dissociation constant of caffeine. If the absorption characteristics of the compounds produced by the addition of alkali or acid are different from those of the "neutral" forms it is possible to calculate the dissociation constants from a knowledge of the variation of the optical density of a solution of the substituted xanthine with the *p*H of the solution.

EXPERIMENTAL

Theophylline, theobromine, and caffeine were recrystallized three times from hot distilled water and dried at 100°. All solutions of these substances contained 20 mg. per liter and were prepared with distilled water. Adjustment of reaction in the range of *p*H approximately 7 to 10 was effected by inclusion of Clark and Lubs buffer components; more strongly acid or alkaline solutions were adjusted by adding hydrochloric acid or carbonate-free sodium hydroxide solution. In every case compensation for absorption by acidifying or alkalinizing agents was made by the use of appropriate blanks.

All spectrophotometric measurements were made using a Model DUV Beckman Quartz Spectrophotometer. Fused silica cells, containing 1.000 cm. thickness of solution, were employed and all measurements were made at a slit width of 0.80 mm. Measurements for the determination of *p*K values were performed at wave length 240 mμ, where a pronounced vertical shift of the absorption curves of the xanthine compounds was observed. *p*H measurements were made before and after spectrophotometric measurement with a Leeds and Northrup *p*H meter, employing glass and saturated KCl-calomel electrodes. The temperature at which all observations were made was 25°.

* Received August 13, 1948, from the Department of Chemistry, Philadelphia College of Pharmacy and Science, Philadelphia 4, Pa.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
† Based upon the thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Chemistry, by Arthur Turner, Jr. (present address, Eastern Regional Research Laboratory, Philadelphia, Pa.).
‡ Professor of Chemistry and Director of the Department of Chemistry, Philadelphia College of Pharmacy and Science.

RESULTS AND DISCUSSION

Figure 1 shows the spectral transmission curves for aqueous solutions of theophylline, theobromine, and caffeine; the recorded *p*H is that of the solution without special adjustment.

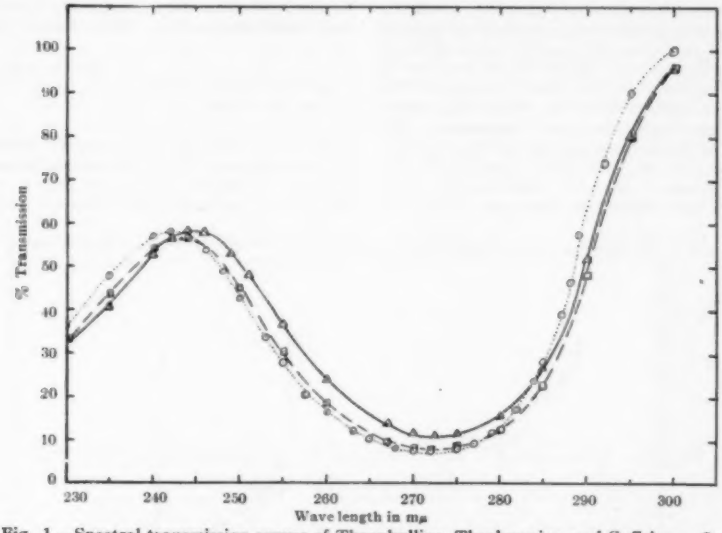


Fig. 1.—Spectral transmission curves of Theophylline, Theobromine, and Caffeine. ⊙, Theophylline *p*H 6.4; ⊡, Theobromine *p*H 5.7; △, Caffeine *p*H 5.6.

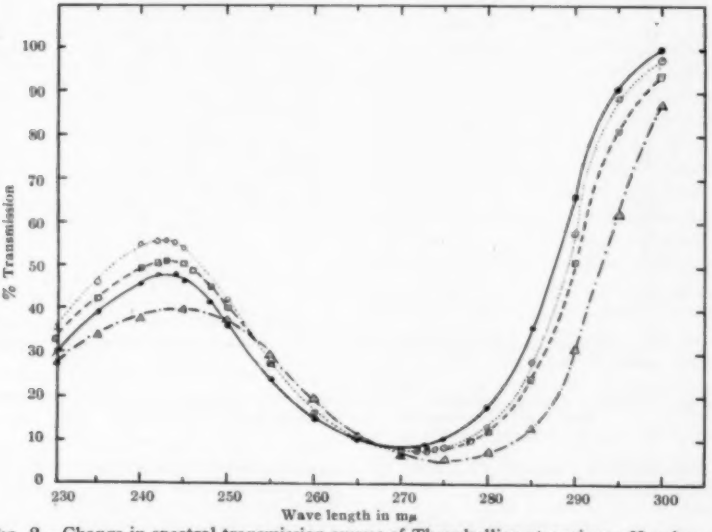


Fig. 2.—Change in spectral transmission curves of Theophylline at various pH values. ●, pH 1.0; ⊙, 6.4; ⊡, 8.6; △, 12.5.

Figure 2 shows the effect of variation of *p*H on the spectral transmission curve of theophylline. It is apparent that a pronounced vertical shift, notably at wave length 240 mμ, occurs. This is due to the different absorption characteristics of the different forms of theophylline obtained by the addition of acid or alkali. Similar curves were obtained for theobromine and caffeine, except that with the latter substance no significant displacement was observed on adding alkali, an observation expected from the

fact that caffeine cannot react as an acid. When conversion to a single entity is complete the absorption curve undergoes no further change upon addition of acid or alkali, as the case may be.

If the optical density of each of a series of solutions of constant concentration but different pH is determined at a wave length where a pronounced spectral shift occurs (in this case 240 mμ), the curves shown in Fig. 3 may be derived. Analysis of these curves in the region representing alkalinized solutions permits quantitative evaluation of the acidic dissociation constants of theophylline and theobromine; the mid-point of the S-shaped portion of the curves for these substances in the alkaline region is the pH at which the form produced by alkali and the "neutral" form are present in equimolar quantities. selected graphically represents the optical density of equimolar quantities of two forms of theophylline and theobromine, respectively, and therefore also the pK_a of each of these xanthines may also be checked by the equation of Beadle and Zscheile (2) for the resolution of two-component systems from spectrophotometric data. Their equation states:

$$\%\ \text{Alkali-produced form of the xanthine} = \frac{(D/C) - k_1}{k_2 - k_1} \times 100$$

where D = optical density at the pH considered to be equal to pK_a, C = total concentration of both forms of the xanthine in Gm. per liter, k_1 = specific extinction coefficient for the "neutral" form of the

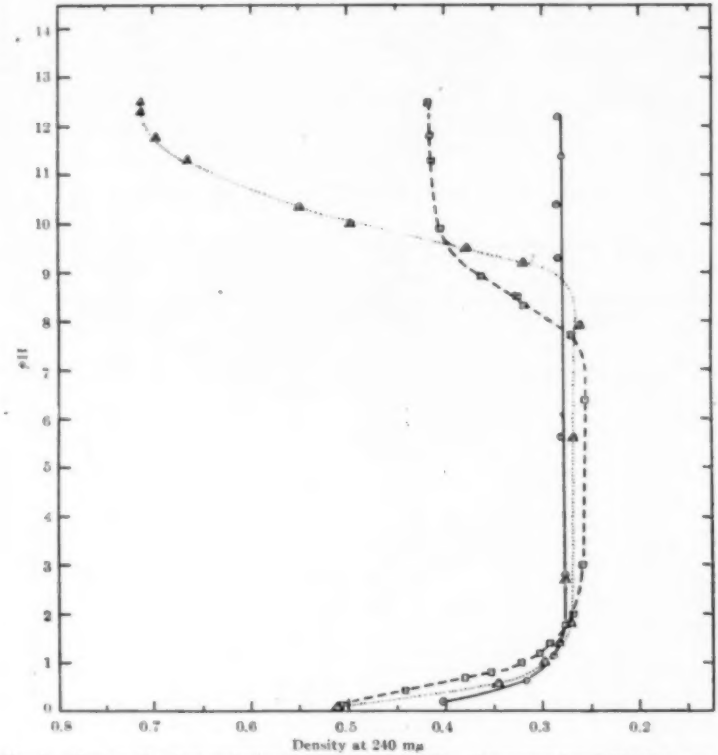


Fig. 3.—Curves showing variation in density with pH. Concentration 0.020 Gm. per liter. △, Theobromine; ⊡, Theophylline; ⊙, Caffeine.

This pH is numerically equal to pK_a, from the fundamental relationship:

$$pK_a = pH + \log \frac{[\text{form produced by alkali}]}{[\text{"neutral" form}]}$$

The pK_a values for theophylline and theobromine, graphically obtained, are 8.6 and 10.0, respectively; these are in good agreement with the values of 8.6 and 9.9, respectively, obtained from potentiometric measurements by Ogston (1). That the mid-point xanthine, and k_2 = specific extinction coefficient for the alkali-produced form of the xanthine. The values for theobromine, namely, $D = 0.505$, $k_1 = 14.1$, $k_2 = 36.4$, and $C = 0.02$, lead to 50.2% of theobromine in alkali-produced form at the mid-point of the curve in Fig. 3. For theophylline the values $D = 0.350$, $k_1 = 13.5$, $k_2 = 21.5$, and $C = 0.02$ lead to a proportion of 50.0% of alkali-produced form of theophylline at the mid-point of its curve.

Since the straight-line portion of the curve for caffeine (in Fig. 3) is not significantly altered in the

alkaline region it may be concluded also from this experiment that the substance does not react as an acid.

Inspection of the curves in Fig. 3 in the region of acidified solutions shows that all three xanthine derivatives undergo reaction with acids beginning at about pH 2. Although measurements were made on solutions having an apparent pH as low as 0.2, the optical density did not attain a constant value, which would have been indicative of complete conversion to a single entity. The most that can be said is that the basic dissociation constants for theophylline, theobromine, and caffeine, respectively, correspond to a pK_b of not less than 13.

SUMMARY

1. The ultraviolet absorption spectra of aqueous solutions of theophylline, theobromine, and caffeine have been investigated, including also a study of the effect of variation of pH on the spectra.

2. From these data the acidic dissociation constants of theophylline and theobromine, expressed as pK_a values, have been found to be 8.6 and 10.0, respectively; caffeine has been found also by this method not to function as an acid. The basic dissociation constants of the three substances correspond to a pK_b value of not less than 13 in any case.

REFERENCES

(1) Ogston, A. G., *J. Chem. Soc.*, **1935**, 1376.
(2) Beadle, B. W., and Zscheile, F. P., *J. Biol. Chem.*, **144**, 21(1942).

Conductometric Studies of Certain Salts of Penicillin*

By FRANK M. GOYAN†

A one-tube balanced amplifier and rectifier for visually detecting the minimum signal of a typical Jones and Josephs conductance bridge is described. This circuit increases the maximum sensitivity of the bridge, but is under complete control of the operator who may select any degree of sensitivity desired. The apparatus described is suitable for work of high precision or for routine conductometric titrations. A technique is developed for conductometric investigation of solutions of salts of benzyl penicillin; the results show graphically the degree of ionization of the different salts studied.

THE WORK of Woodbury and Rosenblum (1) on the conductance of solutions of sodium benzylpenicillinate established the fact that this salt behaves as a completely dissociated electrolyte of the 1:1 valence type at 30° up to a concentration of about 0.05 *M*. Kumler and Alpen (2) confirmed these findings by extending the investigation to include other salts, namely potassium and aminoacridine benzylpenicillinates. Additional work in this laboratory has substantiated further the conclusion that benzylpenicillinate ion in water solution is a typical, large, univalent, negative ion.

While it is entirely possible that certain combinations of ions with the benzylpenicillinate ion in solution may offer decided advantages or disadvantages, in terms of biological activity or otherwise, it is immaterial what pure salts are dissolved to provide the desired concentrations of the various ions under consideration. For example, a solution of potassium benzylpenicillinate and sodium chloride may be identically the same as a solution of sodium benzylpenicillinate and potassium chloride. It is assumed, for the purpose of this argument, that traces of impurities associated with the different solid salts may be eliminated from consideration; however, this assumption is not always justified in practice.

The difficulties involved in preparing any given salt of penicillin suggest the desirability of extending the study of different salts to mixed electrolytes which can be prepared from available pure salt of penicillin by the addition of a second strong electrolyte. For example, the behavior of sodium benzylpenicillinate may be inferred from a study of solutions of potassium benzylpenicillinate and sodium chloride by well-known methods of conductometric titration (3, 4). These titrations involve making many

* Received Sept. 2, 1948, from the University of California, College of Pharmacy, San Francisco 22, Calif.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
† The author takes pleasure in acknowledging the professional and financial assistance of the Cutter Laboratories of Berkeley, Calif.

conductivity determinations, one after each addition of a small uniform sample of a fairly concentrated solution of one of the electrolytes of the mixture. However, before proceeding with this general method, it was necessary to overcome technical difficulties which evidently have hindered a more general application of conductance measurements to the solution of problems involving substances of pharmaceutical interest. Studies of this nature often require a precision of measurement somewhat greater than is easily obtained with one of the instruments designed exclusively for conductometric titrations (5).

It is extremely arduous to undertake many routine measurements involving the use of a bridge method requiring detection of the point of balance by estimating a condition of minimum sound in earphones. As a matter of fact, the author is convinced from his own personal experience that there is a very real element of danger to the operator in using audio methods because of the temptation to increase amplification well above room noise level at the time the point of bridge-balance is reached. As a result of this excessive amplification, there is a tendency to allow dangerously loud vibrations to reach the ear when the bridge is accidentally thrown out of balance. To be sure, this danger may be overcome to a large degree by proper amplifier design; however, it is desirable to make use of visual methods of detection if possible.

The design of a balance detector of the visual type to be used with existing facilities, consisting of a Jones and Josephs type bridge with electronic oscillator and amplifier originally intended for audio detection, was so successful that it became clear that routine measurements of electrical conductance with any desired degree of precision were entirely practicable. In fact, the circuit developed to meet this need, presented schematically in Figure 1, makes it possible to increase the precision of the existing bridge by a factor of ten, when desired, without sacrificing the ease or speed of attaining approximate balance within predetermined limits. There is, of course, the usual psychological problem arising from the fact that an interested operator is seldom content with less than the maximum precision of the instrument. However, this problem can be overcome by adjusting the work to a time schedule.

EXPERIMENTAL

Standard Electrical Equipment.—The conductivity bridge used in this work was constructed from component parts purchased over a period of years.

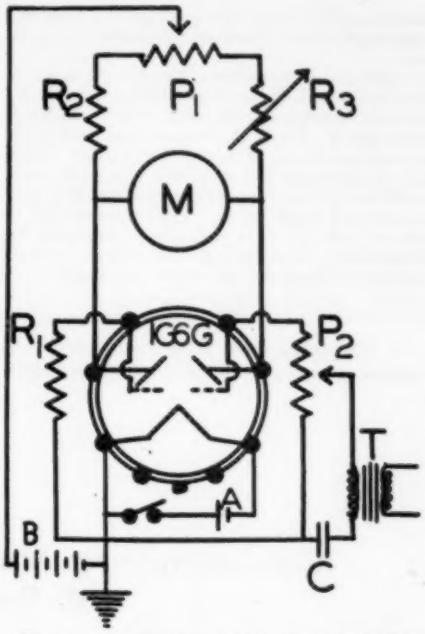


Fig. 1.—Visual Balance Indicator Circuit Diagram. *A*, 1.5-v. "A" battery; *B*, 90-v. "B" battery; *C*, 0.005 m. f. d. or larger; *M*, microammeter (0–100 microamp. with six-inch dial used but not required); P_1, 500 ohms; P_2, 1 megohm; R_1, 1 megohm; R_2, 7000 ohms; R_3 0–10,000 ohms.

Up to the time of the introduction of the visual balance indicator, the assembly of a General Radio Ratio Arm Box (type 610-A), a six-dial Leeds and Northrup 10,000-ohm decade resistance box and vacuum tube oscillator (Cat. No. 9842), together with necessary resistors and condensers, represented a typical Jones and Josephs bridge (6) with a Wagner earthing device. A vacuum tube amplifier of conventional design was coupled to the bridge by means of a suitable impedance matching transformer. This circuit has remained unchanged with the introduction of the visual balance indicator.

Visual Balance Indicator.—The new visual balance indicator was designed to replace the phones in the existing circuit. Instead, it replaces one of a pair of phones; the other phone remains in series with the primary of the transformer T (Fig. 1). This arrangement has proved to be desirable in teaching; the single phone is never placed near the ear, but it is easily heard when the bridge is far off balance. Because of the fact that the amplifier and oscillator are powered from the 115-v. a.-c. source, it was decided to power the visual balance indicator with batteries to avoid possible difficulties with the ground connections. The filament of the 1G6G tube (Fig. 1) operates for many hours from one 1.5-v. No. 6 dry cell. Two 45-v. radio B batteries in series supply the plate voltage. The size of the B batteries is not critical because the total plate current drain is less than 3 ma. The meter M

(Fig. 1) is a six-inch dial Triplett microammeter (range 0–100 microamperes). This meter is very convenient because it is easy to see from any position near the instrument, but any galvanometer of equal or greater sensitivity will serve the purpose. The potentiometers, P_1 and P_2 are the principal controls: R_3 is adjusted so seldom that it need not be placed on the front panel. The transformer T is an old audio frequency transformer with a 3:1 ratio introduced to provide insulation from unwanted ground connections of the amplifier circuit rather than for critical impedance matching. Condenser C was introduced with a similar purpose in mind, but the circuit seems to work well when this condenser is shorted.

Shielding.—Various components of the entire bridge seem to be critical with respect to position. For example, it is necessary to place the input transformer a foot or more away from the amplifier because of the power transformer built into the amplifier cabinet. All of the metal cases of the various components of the bridge are grounded, and some of the lead wires are shielded. Shielding is less critical with the visual balance indicator because of the fact that it is possible to observe the point of minimum deflection against any steady background noise. However, reduction of noise level is important when maximum sensitivity is demanded. Considerable loss of efficiency is noted when certain fluorescent lamps are operated on the same table. A tuned amplifier or band-pass filter is indicated but has not as yet been added to the circuit.

Thermostats.—An oil thermostat is available for special work; however, for reasons of convenience, a water thermostat was used for this work. The thermostat is maintained at 25° within 0.01° by means of a mercury regulator and relay. On warm days an air blower playing over the surface of the water serves to prevent loss of control. Work at or near 0° is done in a vacuum jar filled with chopped ice and covered with a fitted plastic box also filled with ice.

Titration Cell.—A Leeds and Northrup Henry type conductivity cell (7) with lightly platinized electrodes is used as a titration vessel. A cell constant of 0.208 cm.$^{-1}$ was determined for the cell when filled with 0.001 M KCl to a predetermined mark representing a volume of 26.7 cc. Stirring after each addition of electrolyte is accomplished either by bubbling washed nitrogen from a glass capillary which must be removed before balancing the bridge, or by means of a glass propeller-type stirrer rotating near the surface of the liquid. The glass stirrer operates well above the electrodes and need not be removed to balance the bridge.

Operation of the Bridge.—Resistance measurements of the filled cell are made by conventional methods involving selection of suitable ratio-arm settings followed by adjustment of the Wagner earthing device, and finally by adjustment of the decade resistance box to position of bridge balance. The amplifier is permanently set to a high stable value; control of sensitivity is achieved by means of the oscillator volume control and the potentiometer P_2 (Fig. 1). When the visual balance indicator is first put into service R_3 is adjusted so as to cause the meter to read between zero and midscale, while P_1 is near the center of its range and P_2 is at its ground or insensitive position. The meter is then set to zero with P_1, and finally P_2 is adjusted to give a high meter reading. As the balance point is reached the meter reading approaches zero. When additional sensitivity is required, P_2 is further adjusted toward higher sensitivity, and more power is supplied from the oscillator until the meter again reads a high value. Further adjustment of the bridge will cause the meter reading again to approach zero. The lowest meter reading indicates the point of best bridge balance. For rapid conductometric titrations it is desirable to arrange the sensitivity of the visual balance indicator so that the only sensitivity control needed is the volume control of the oscillator which was operated at 1000 cycles per second.

Titration Techniques.—All electrolytes studied were made up in distilled water to a concentration of 0.1 N. The titration vessel was filled to the predetermined mark with redistilled water which was checked to avoid the possibility of contamination at the start of each titration. The first electrolyte was added to the water from a micro-pipette designed to deliver 0.1 ml. After each addition, the bridge was balanced and the resistance recorded. After the addition of 0.4 ml. of 0.1 N solution of the first electrolyte, the second electrolyte was added using the same pipette, and resistance measurements were made after each addition until 0.7 or 0.8 ml. had been added. Care was taken to maintain uniformity of volume and of concentration, but little attention was paid to absolute accuracy. The potassium benzylpenicillinate used was of good quality but not the best available (1500 units per milligram), and the other salts were of C. P. or reagent quality. The mercuric nitrate was dissolved in 0.02 N nitric acid to prevent precipitation of basic salts. The solutions were made with some care but only the potassium chloride was dried before weighing. The concentrations of the other solutions may be less than the nominal value, a matter of no importance in the present study.

RESULTS

The results of several conductometric titrations are presented graphically in Fig. 2. In all cases the reciprocals of the measured resistances in ohms were read from an ordinary ten-inch slide rule and plotted against the volume of solution added. The curves shown are sufficient to illustrate the method. It will be noted that only the hydrochloric acid curve and the mercuric nitrate curve show marked curvature. The formation of a moderately weak acid is to be expected and is accounted for by the observed shape of the curve. The titration with mercuric nitrate caused the formation of a white precipitate that failed to redissolve upon the addition of more reagent or of nitric acid. The nature of this precipitate cannot be deduced with certainty from the curve, but it is interesting to note that the straight line portion begins at the point corresponding to chemically equivalent amounts of mercury and pencillin. This would suggest a salt of the type, HgP_2, where P represents the univalent benzylpenicillinate radical.

The fact that the other curves approach straight lines in those portions representing the addition of the second electrolyte (0.4 cc. except for the HCl

curve which begins at 0.5 cc.) indicates that the corresponding salts are normal strong electrolytes.

DISCUSSION

It can be shown from the fundamental definitions of equivalent conductance and specific conductance (7) that the reciprocals of resistance values (obtained with the same conductivity cell and the same electrolyte in solution) are proportional to concentration of the electrolyte if its equivalent conductance remains constant. Actually there are two compensating errors that make the points shown in Fig. 2 fall on straight lines as nicely as they appear to do. The equivalent conductance values are not strictly constant, and volume of added reagent is slightly different from a linear function of concentration because, with each addition of reagent, there is a corresponding dilution of the entire solution due to

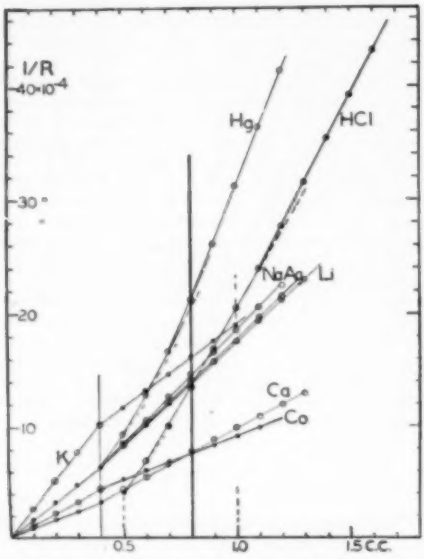


Fig. 2.—Results of Conductometric Titrations. Small solid points represent addition of fresh 0.1 *N* solution of potassium benzylpenicillinate. Larger circles represent 0.1 *N* solutions of different salts as follows: At 0° C. Ca = $CaCl_2$, Co = $CoCl_2$, HCl = HCl. At 25° C. K = KCl, Na = NaCl, Ag = $AgNO_3$, Li = LiCl, Hg = $Hg(NO_3)_2$ in 0.02 *N* HNO_3. (Initial volume of water = 26.7cc.)

the slight increase in volume, superimposed, of course, upon the larger increase in concentration.

Although it is not difficult to compensate for the dilution effect in plotting, thereby permitting a calculation of equivalent conductance within the limits of volumetric accuracy at any concentration, this is not done because it is felt that the principal value in this method lies in its simplicity. The slope of any line is taken as an empirical measure of the equivalent conductance of the electrolyte being added or being formed by chemical interaction. In the case of titration with hydrochloric acid, if the weak acid formed were a very weak electrolyte, the curve would have the slope shown for potassium chloride. If no weak acid were formed, the slope of the HCl line between the 0.5-cc. point and the 1.0-cc. point would be the same as that of hydrochloric acid shown beyond, and there would be no break in the curve. Although later work may demonstrate the value of more exact analytical treatment of the data, the principal interest at the present time is to discover abnormalities in solutions that may suggest need for further investigation in a broader sense than might be attempted by refinement of conductance measurements.

SUMMARY

The study of certain salts of benzylpenicillinate is undertaken by a method of conductometric titration. Results are presented graphically showing that lithium, sodium, potassium, silver, calcium, and cobalt salts of benzylpenicillinate show no marked deviation from the behavior of strong electrolytes in dilute aqueous solutions. The formation of a weak acid is verified by the curves presented, and the formation of a white precipitate of some mercury salt is reported and correlated with the corresponding curve.

The potential serious danger to the hearing of operators of sensitive bridges depending upon detection of balance by estimating minimum sound in phones is mentioned as one reason for the development of a visual balance detector that may be used to improve the sensitivity of a typical Jones and Josephs bridge. Circuit details are given and directions are included for using the new visual method with any degree of precision desired. The visual method of detection of balance may be used for rapid approximate measurements because the point of balance can be detected over background noise.

REFERENCES

(1) Woodbury, D. T., and Rosenblum, C., *J. Biol. Chem.* **171**, 447(1947).
(2) Kumler, W. D., and Alpen, E. L., Private communication.
(3) Weissberger, A., "Physical Methods of Organic Chemistry," Interscience Publishers, Inc., New York, 1946 Vol. II, p. 1034.
(4) MacInnes, D. A., "Principles of Electrochemistry," Reinhold Publishing Corporation, New York, 1939, p. 382.
(5) Garman, R. L., *Ind. Eng. Chem., Anal. Ed.*, **8**, 146 (1936).
(6) Jones, G., and Josephs, R. C., *J. Am. Chem. Soc.*, **50**, 1049(1928).
(7) Lyman, R. A., "American Pharmacy," J. B. Lippincott Co., Philadelphia, 1947, Vol. II, p. 106.

Routine "Safety" Tests on Drug Products and the Organization and Maintenance of a Requisite Mouse Colony*

By JOHN H. BREWER†

ALTHOUGH required by the National Institute of Health for most of the biological products manufactured under their license, many injectable preparations on the American market are not subjected to routine safety testing in animals. On many of these products the manufacturer depends entirely on routine analytical and sterility tests, and the final pharmaceutical preparation is not actually tested for toxicity before being released for sale.

In our manufacturing processes we have believed that a routine safety test would give us a feeling of security and also be a check on our manufacturing, sterility, and control laboratories. We have determined for each ampul preparation manufactured by us, based on the human dose, the human equivalent dose for white mice. We record in our testing the reaction in mice to the human equivalent dose. We also determine the maximum dose which can be given to 20-Gm. white mice without producing death. From this is calculated and recorded the "Safety Factor" which represents the magnitude of the dose as compared to the equivalent human dose. The products are injected either intravenously by tail vein injection or intraperitoneally in the case of oil preparations. Ten ampuls from each lot manufactured are submitted to these tests and the animals are observed for seven days. This, along with other tests, is released on the same day as our routine sterility test and thereby does not hold up the production of any ampuled product, unless unwarranted toxicity is found.

TESTS APPLIED

For some of our products, for instance BAL in Peanut Oil, we can give only 9.75 times the equivalent human dose without reaction. In the case of other preparations, however, the entire human dose may be given, which in some instances is 3500 times the human equivalent dose in mice, without any sign of reaction. For instance, in the case of phenolsulfonphthalein, the human equivalent dose would be 1 cc. of a 1:3500 dilution of the phenolsulfonphthalein. This produces no reaction in mice. In our routine animal safety tests, however we give 1 cc. of the full strength material. This is 3500 times the human equivalent dose and produces no reaction in mice.

The route of injection is by tail vein and since this is the actual human dose, the lethal dose is not determined. In the case of many preparations which produce death, however, we do determine such minimal lethal dose (or LD_{m}) on each lot, or rather determine that this *M. L. D.* for any particular lot is not greater than the average for this particular product. Bromsulphalein is a good example of this type preparation. The average human dose ampul size is 3 cc. The equivalent human dose in mice is 1 cc. of a 1:1150 dilution. This dosage produces no reaction in mice. In our routine tests, however, we administer 0.1 cc. of the full strength material intravenously and this is 115 times the equivalent human dose based on a 2 mg./Kg. dosage. It would be 46 times the equivalent human dose if 5 mg./Kg. were used. This material is given slowly and very carefully to produce no necrosis and no reaction in the animal. However, if injected rapidly so that some of the material goes intrastitially, it may cause necrosis or even death of the animal. Lethal dose of this material for 20-Gm. white mice is 0.18 cc. of the full strength material. A typical chart of these products is shown in Table I.

This type of testing simulates the safety test required by the National Institute of Health for products made under its license and gives a feeling of security concerning our preparations which we would not otherwise have, since it is entirely possible that a product may pass the routine sterility tests and also chemical analysis, and still cause unfavorable reactions when injected, particularly in the case of some of the products whose therapeutic index is very low. In addition, such tests serve to eliminate any lots which might conceivably produce reactions from other causes not connected with the drug itself. These tests can be conducted at the same time the other routine tests are done and do not hold up our production in any way.

Mouse Colony.—The mouse colony described is small but practical and its primary purpose is to supply a sufficient number of mice to be used for routine safety and toxicity tests. Proper equipment is possibly the most important factor in a successful mouse colony. Stainless steel is the ideal material for all units. Aluminum may also be used advantageously. However, if aluminum is used, one must make all openings small so that the mouse cannot get his teeth through and gnaw his

* Received February 2, 1949.

† Director of Biological Research, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

The author wishes to thank Mr. Harold Bryant for suggestions and ideas relative to the cage and box design, and Mr. Raymond Bullinger for the information relative to the operation of the colony.

The stainless steel equipment was manufactured by the Baltimore Biological Laboratory.

TABLE I.—ANIMAL CONTROL TESTS OF STERILE PRODUCTS

Product	Human Dose (According to Ampul Size)	Animal Dose (Mice) (Equivalent of Human Dose)	Reaction in Mice to Human Equivalent	Dose Administered in Routine Test	Routine Test Is Number of Times of Human Dose	Reaction to Routine Dose	Route of Injection	Lethal Dose
BAL in Peanut Oil	4.5 cc.	1 cc. of 1:780 dilution	No reaction	0.25 cc. of 1:20 dilution	9.75 times	No reaction on injection	Intraperitoneally	0.5 cc. of 1.20 dilution
Phenolsulfonphthalein	1 cc.	1 cc. of 1:3500 dilution	No reaction	1 cc. full strength	3500 times	No reaction	Tail vein	Not determined
Bromsulphalein	3 cc. (2-mg. dose)	1 cc. of 1:1150 dilution	No reaction	0.1 cc. full strength	115 times	Give slowly; may cause necrosis in tail	Tail vein	0.18 cc. full strength

way out. The dimensions and figures given below have been obtained from our colony and could be changed to suit other conditions without altering the utility.

The breeder box (Fig. 1, *A*), as used in this colony, measures nine and one-half inches wide, twenty inches long, and four and one-half inches deep. This is fitted with a perforated top (Fig. 2, *A* and *B*), which is designed to hold a wire mesh feeder that will extend through to the underside, and a rack to hold water bottles, the ends of which will also extend through to the underside. These units can be fed and watered from the top without removal of the lid, thereby greatly reducing the time required for the daily feeding and watering of the colony.

A practical stock box (Fig. 1, *B*), which is used in conjunction with the breeding colony, measures seven inches wide, ten inches long, and six and one-half inches deep; it is fitted with a wire mesh top which contains the wire mesh feeder and the water bottle rack (Fig. 2, *C* and *D*). This box is not only used to house the stock mice weaned from the breeder units but also for the mice during the tests.

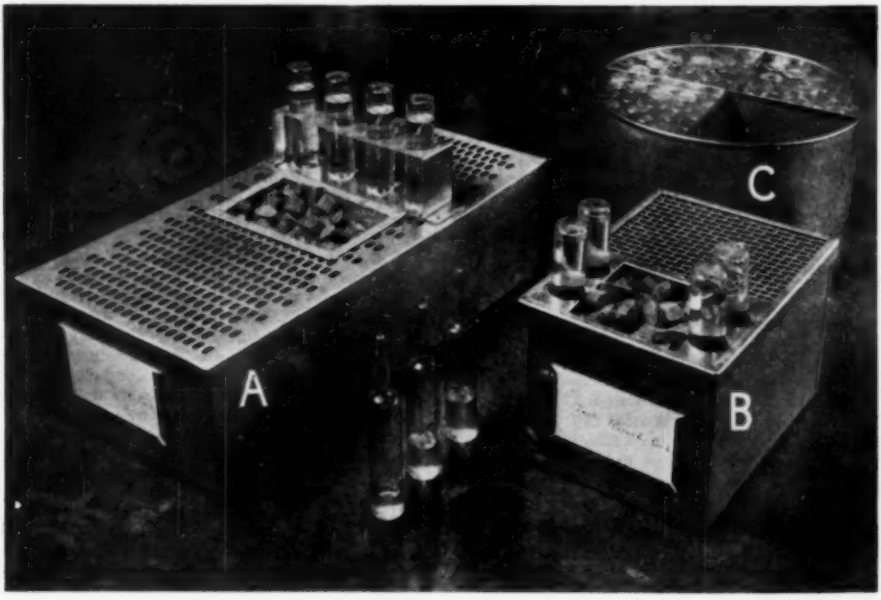


Fig. 1.—Breeder box, stock box and sterilizer pan.

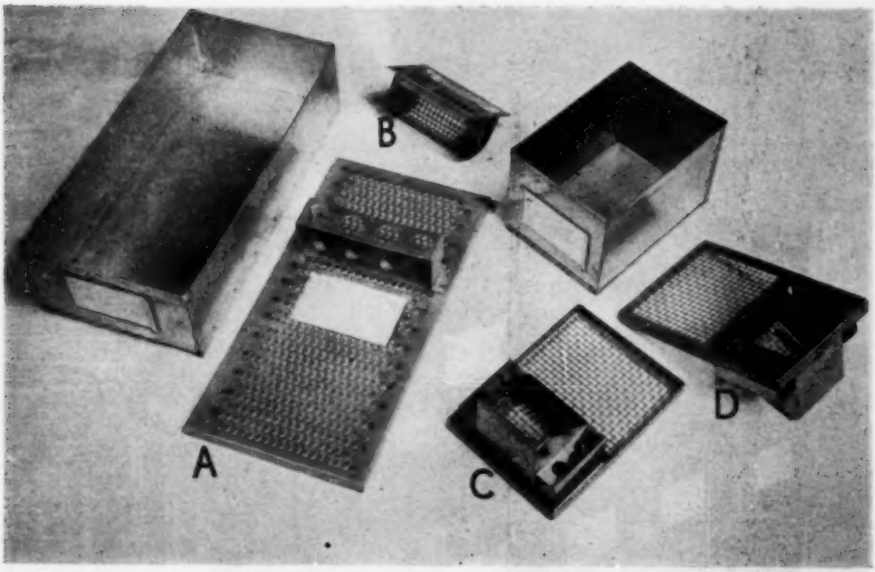


Fig. 2.—Expanded view of breeder box and stock box.

Fig. 3.—Breeder units.

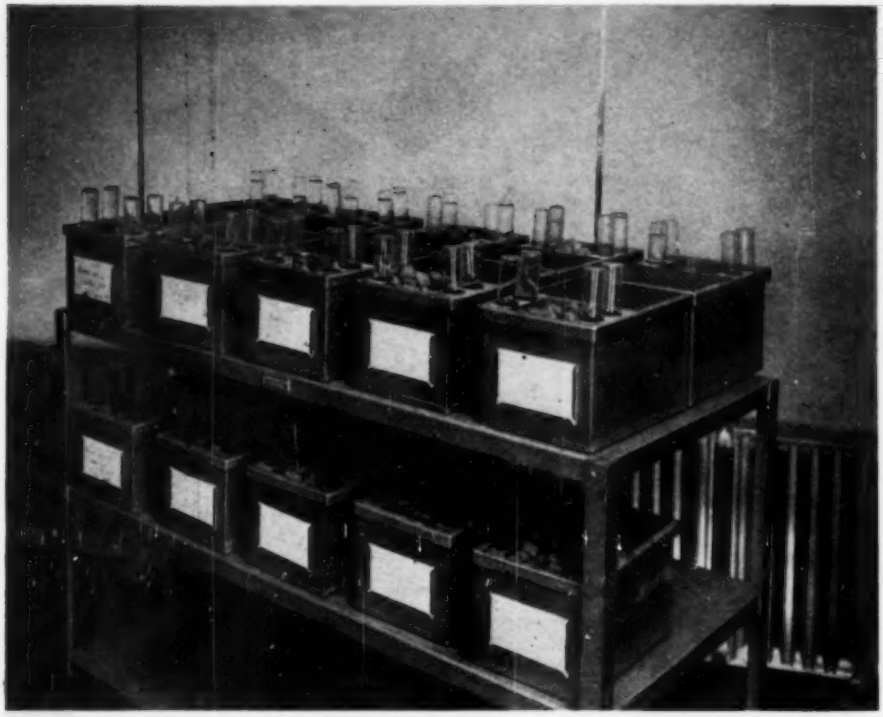


Fig. 4.—Stock boxes and mice under test.

The water bottle mentioned above is a standard (Kimble) fifty milliliter ampul with the mouth flamed in such a manner as to produce a small opening (3.5 mm.). When inverted into the rack, clean water is readily available to the animals merely by licking the lip of the ampul. Four of the ampuls are supplied to each breeder unit. The water can be automatically filled into the ampuls and sterilized in one operation with the use of suitable pans (Fig. 1, *C*) and an autoclave. The pans, containing the empty, inverted ampuls are partially filled with water and placed into the autoclave. The steam pressure is raised to twenty pounds and held there for twenty minutes. The amount of water to be filled into the pans is governed by holes in the pan about two inches from the top. Upon cooling, a partial vacuum is formed in the ampuls and this space is in turn filled with the water in the bottom of the pan as the ampuls cool.

Based on individual experiments, it has been estimated that the average mouse will drink approximately four milliliters of water and eat four grams of food daily (Purina Lab. chow).

The breeder units are kept on a four-shelved dolly (Fig. 3); each shelf is large enough to accommodate four breeder units. This comprises a sixteen unit "group." Six of such "groups" are in operation in this model colony. The stock boxes and mice under test are kept on similar dollies (Fig. 4).

Both the stock and breeder boxes are furnished with about one-half inch of fine sawdust for bedding. All boxes are washed in a warm chlorine solution twice a week and fresh sawdust supplied.

The time requirements for the care and maintenance of this colony are as follows: changing stock and breeder boxes, washing boxes, and replacing sawdust—two persons two and one-half hours twice a week; daily feeding and watering, one person approximately two hours; weaning of young, one person approximately one-half hour per group once a month.

Now that the proper equipment has been procured and arranged in a suitable manner, it is a relatively simple matter to establish the breeding colony. Seven females and one male comprise the family and a family is placed in each of the breeder units previously mentioned. The young from these units are weaned once a month, a good average yield being ten. However, this number may vary from five to twenty-five or more, depending on the age and condition of the breeders and the correlation of the gestation cycles.

The colony mentioned above consists of six groups in three pairs, a total of ninety-six breeder units. Each pair of groups, or thirty-two units, is weaned once a month. When at the peak of production, this colony will produce approximately two hundred usable mice per week. If the production of any one unit falls off considerably, it is

advisable to replace the entire unit with new mice because the malfunction of this one unit will naturally bring down the average of the whole group. The highest productive age span for the average mouse is from two to ten months; after ten months the production drops off and it is well to replace the unit with young mice.

A very important factor is the temperature of the room in which the colony is housed. This temperature must be fairly constant, ranging from 75° to 80° F. If this temperature is not maintained, serious damage or death may result to the breeding animals and the average output of young is considerably lessened.

Although the stainless steel equipment shown in the accompanying photographs is relatively expensive when compared with galvanized boxes, it will outlast several sets of galvanized cages and withstands sterilization and chemical agents without corrosion or discoloring.

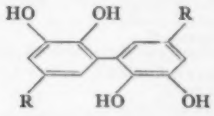
The Synthesis of Some Effective Antioxidants and Antiseptics*

By AKIRA ASANO† and OLE GISVOLD‡,§

Certain guaiacol derivatives can be readily coupled to yield the corresponding dehydrobicompounds which in turn can be readily converted to the corresponding dehydrobicatechols. Therefore, the following 2,2′,-3,3′-tetrahydroxy biphenyls were synthesized, i.e., 5,5′-dimethyl (I); 5,5′-di-*n*-propyl (II); 5,5′-di-*n*-amyl (III); and the methyl (IV), ethyl (V), and normal propyl (VI) esters of the 5,5′-dicarboxy compound. Compounds I, II, and VI exhibited very powerful bacteriostatic activity that was inhibited by blood serum. The alkyl derivatives I, II, and III exhibited marked antioxidant activity in lard when tested by the Swift rapid rancidity method. The esters IV, V, and VI gave poor protection.

It is known that eugenol easily undergoes oxidative coupling with ferric chloride to yield dehydrodieugenol (2,2′-dihydroxy-3,3′-dimethoxy-5,5′-diallyl biphenyl). This type of coupling with eugenol was first reported in 1908, by Cousin and Herissey (1), who investigated the dehydrogenation of eugenol with an oxidizing enzyme and with ferric chloride. Certain other phenolic compounds also undergo oxidative coupling with various oxidizing agents.

Nordihydroguaiaretic acid (2, 3) and alpha, omega-bis(3,4-dihydroxyphenyl) alkanes (4) have been shown to possess marked antiseptic and antioxidant properties. This suggested the synthesis of 4,4′-disubstituted 2,2′,3,3′-tetrahydroxy biphenyls as possible useful antiseptics and antioxidants. Since 4-substituted guaiacols, such as eugenol and vanillin, couple in the unoccupied orthoposition to the hydroxyl group to form the corresponding bis compounds, the preparation of substituted dehydrobicatechols was undertaken by first coupling certain 4-substituted guaiacols. The following substituted dehydrobicatechols were prepared:



where R = CH_3, $CH_2CH_2CH_3$, $CH_2CH_2CH_2CH_2CH_3$, $COOCH_3$, $COOCH_2CH_3$ or $COOCH_2CH_2CH_3$.

Very few biphenols have been reported for their antiseptic or antioxidant purposes. 2,2′-Dihydroxy biphenyl and its derivatives were investigated for their fungicidal activity by Marsh and Butler (5), and 4,4′-dihydroxy biphenyl has been patented as an antioxidant (6).

In preliminary tests the dehydrobicatechols showed varying degrees of antioxidant activity by the Swift Stability Test using 0.005 per cent concentrations in lard. The alkyl substituted dehydrobicatechols showed good antioxidant activity on lard. On the *w/w* basis the activity of the alkyl substituted compounds decreased as the alkyl chain increased. A more rational comparison would be on a molar basis. The esters of

---

* Received August 30, 1948, from the College of Pharmacy, University of Minnesota, Minneapolis.
Presented to The Scientific Section, A. PH.A., San Francisco meeting, August, 1948.

† Abstracted in part from a thesis submitted by Akira Asano to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.

§ The authors wish to thank Dr. K. K. Chen and the Bacteriology Department of Eli Lilly and Company for the antiseptic tests.

dehydrodiprotocatechuic acid showed very little activity. The results of the test are shown in Table I.

The results of the preliminary antiseptic test upon *Streptococcus viridans* using a synthetic chemical medium consisting of amino acids, vitamins, sugar, and salts are tabulated in Table II. Further investigation upon 2,2′,3,3′-tetrahydroxy-5,5′-dimethyl biphenyl; 2,2′,3,3′-tetrahydroxy-5,5′-di-*n*-propyl biphenyl; and di-*n*-propyl ester of 2,2′,3,3′-tetrahydroxy-5,5′-dicarboxy biphenyl showed phenol coefficients on *Streptococcus viridans* of 1600, 800 and 3200, respectively. These three most active compounds showed no activity against *Staphylococcus aureus* and *Bacillus typhosus* using concentrations as great as 1:50,000 in beef extract medium.

The reason for the great activity originally observed and apparent inertness in further experiments therefore was sought. Using the chemically defined medium, it was found that 1 per cent blood serum prevented the bacteriostatic action of these compounds against *Streptococcus viridans*. Inositol had no effect although it had slight structural similarity to these compounds.

TABLE I

0.005% of Compound	Hr. for Rancidity	Protective Index
2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl	51	6.0
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-propyl Biphenyl	35	4.1
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl	28	3.3
Dimethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl[a]	10	1.2
Diethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl[a]	15.5	1.8
Di-*n*-propyl Ester of 2,2′,-3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	12	1.4
Nordihydroguaiaretic Acid	36	4.2
Control	8.5	...

[a] Five milligrams of the compound were first dissolved in 0.5 cc. of propylene glycol, and the solution then added to the melted lard.

TABLE II

	No Growth	Growth
2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl	1:1,280,000	...
2,2′3,3′-Tetrahydroxy-5,5′di-*n*-propyl Biphenyl	1:1,280,000	...
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl	1:32,000	1:64,000
Diethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	1:80,000	1:160,000
Di-*n*-propyl Ester of 2,2′,-3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	1:1,280,000	...
Phenol	1:1000	1:5000

The reversal by such a small concentration of serum would indicate that they are not of value for sterilization of the skin, wounds, etc. The three most active compounds were also tested against influenza virus, *Streptococcus hemolyticus*, and tetanus toxin *in vivo*. The compounds were ineffective.

EXPERIMENTAL

Dehydrodieugenol or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-diallyl Biphenyl

Dehydrodieugenol previously has been prepared by a procedure that proved very unsatisfactory. The following procedure was devised and gave satisfactory results.

Twenty grams of eugenol was dissolved in 16 L. of distilled water, and to it was added, dropwise, a ferric chloride solution (33 Gm. of $FeCl_36H_2O$ in 250 cc. of water). The mixture was subjected to vigorous mechanical agitation during the addition of ferric chloride, which required approximately two hours. The agitation was continued for four hours, and a brown precipitate, which was obtained, was collected by filtration. About 13 Gm. of crude dehydrodieugenol was obtained. The precipitate was dissolved in 100 cc. of ether and then treated with 5% sodium hydroxide solution until no further precipitation occurred. The entire mixture was heated on a steam bath to remove the ether. The resultant mixture was filtered, and the precipitate was washed with 5% sodium hydroxide solution until the color of the washings became light yellow. Approximately 1500 cc. of 5% sodium hydroxide was necessary. The free phenol was liberated from the sodium salt by boiling the salt in 200 cc. of 10% hydrochloric acid. The mixture was cooled, and the precipitate filtered and washed with water. The collected product was then dried and extracted in a Soxhlet Extractor with petroleum ether (Skelly B) Dehydrodieugenol was only slightly soluble in petroleum ether, and upon prolonged extraction the dehydrodieugenol separated from petroleum ether in the flask. The solvent was decanted, and 10.5 Gm. of a semipure product which possessed a slight yellow color was obtained. This product was crystallized from 95% alcohol after partial decolorization with charcoal. An over-all yield of 30% dehydrodieugenol having a melting point of 101–103° (reported 106°) was obtained. This product was used for the next reaction. Through repeated recrystallizations it was possible to raise the melting point to 104–106°.

Tetrahydrodehydrodieugenol or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-di-*n*-propyl Biphenyl

Hydrogenation of the side chains of dehydrodieugenol was readily accomplished along the lines used by Erdtman. Four grams of dehydrodieugenol was dissolved in 350 cc. of 95% alcohol in a thick-walled bottle, and to it was added 0.1 Gm. of platinum oxide. The reaction bottle was connected to the Parr hydrogenation apparatus. After the introduction of 30 pounds of hydrogen, the bottle was shaken for one hour at room temperature although the hydrogenation appeared to be complete in ten minutes. The reduction product precipitated out. The content of the bottle was trans-

ferred into a beaker and heated to dissolve the reduced compound. The hot solution was filtered to remove the catalyst, and upon cooling tetrahydrodehydrodieugenol crystallized out from the filtrate. Three grams of product melting at 148–150° (reported 152°) was obtained.

By using the mother liquor as a solvent for the subsequent reduction of a second quantity of dehydrodieugenol, hydrogenation became slower and less complete. However, if the reaction mixture was heated to about 60° and then hydrogenated as before, a nearly quantitative amount of tetrahydrodehydrodieugenol was obtained. The mother liquor became slightly colored on repeated use, so it was decolorized with charcoal before each hydrogenation.

Nortetrahydrodehydrodieugenol or 2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-propyl Biphenyl

Five grams of tetrahydrodehydrodieugenol, 25 cc. of glacial acetic acid, 5 cc. of acetic anhydride, and 25 cc. of 48% hydrobromic acid were refluxed for two hours. The reaction mixture was allowed to cool and poured into 250 cc. of water. The reaction flask was rinsed with water and the whole combined. The precipitate that was collected by filtration amounted to 4.0 g. of crude nortetrahydrodehydrodieugenol. This compound was crystallized several times from dilute alcohol containing a small amount of sodium hydrosulfite. The yield was 2.5 Gm. or 59.3% of the theoretical. Melting point, 149–150°.

Anal.—Calcd. for $C_{18}H_{22}O_4$: C, 71.50; H, 7.34. Found: C, 71.74; H, 7.44.

The tetra-acetate was prepared by refluxing nortetrahydrodehydrodieugenol with pyridine-acetic anhydride solution. Melting point, 82.5–84.5°.

Anal.—Calcd. for $C_{26}H_{30}O_8$: C, 66.37; H, 6.42. Found: C, 66.56; H, 6.63.

Dehydrodivanillin or 2,2′dihydroxy-3,3′-dimethoxy-5,5′-dialdoxy Biphenyl

Dehydrodivanillin was prepared according to the directions of Elbs and Lerch (7) with slight modification in the purification. The crude washed product was suspended in 3 L. of boiling water, and the product was dissolved by the addition of 5% sodium hydroxide solution. Five per cent hydrochloric acid was added to this boiling solution to precipitate dehydrodivanillin. The precipitate was filtered and washed with 2 L. of boiling water. After repeating this purification process once more, a light gray product decomposing at 297–300° (reported 303–305°) was obtained in about 82% yield. This product was sufficiently pure for the preparation of dehydrodicreosol and dehydrodiprotocatachuic acid.

The purification method of Elbs and Lerch (7) did not raise the decomposition temperature. By Erdtman's purification method (8), a product decomposing at 300–303° was obtained.

The decomposition temperature for this compound was determined by preheating the melting point bath to 290° and by heating the bath at the rate of three degrees per minute.

Dehydrodiprotocatechuic Acid or 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl

The procedure of Tiemann (9) was used for the preparation of dehydrodiprotocatechuic acid. A yield of 6.2 Gm. of a light pink product was obtained from 10 Gm. of dehydrodivanillin. It decomposed at 306–310° (reported over 300°). This compound was difficultly soluble in the common polar and nonpolar solvents.

Di-*n*-propyl ester of dehydrodiprotocatechuic Acid

Two grams of dehydrodiprotocatechuic acid was suspended in 75 cc. of *n*-propyl alcohol which had been dried over "Drierite." Upon saturating the chilled suspension with dry hydrogen chloride the mixture became a pasty mass. This mixture was heated under a reflux until the solution was effected, which required approximately one hour, and the heating was continued for another hour. Excess alcohol and hydrogen chloride were removed under reduced pressure on the water pump, and 100 cc. of water was added to the residue. The product, collected by filtration, was shaken intermittently for an hour with 100 cc. of 5% sodium bicarbonate solution. The mixture was filtered and the solid residue washed with water. Two crystallizations from dilute propyl alcohol gave 1.6 Gm. or about 62.5% of a product that melted at 220–222°. A sample for analysis was recrystallized several times from dilute propyl alcohol. Light pink crystals melting at 221.5–223° were obtained.

Anal.—Calcd. for $C_{28}H_{22}O_8$: C, 61.53; H, 5.68. Found: C, 61.65; H, 6.03.

A tetra-acetate was prepared as follows: A solution of 0.3 Gm. of di-*n*-propyl ester of dehydrodiprotocatechuic acid in 5 cc. of acetic anhydride and 10 cc. of dry pyridine was refluxed for two hours, and then cooled to room temperature. The solution was poured into 50 cc. of ice water with constant stirring. The tetra-acetyl derivative which had separated was filtered, washed with 50 cc. of water, and then shaken for an hour with 25 cc. of 1% sodium hydroxide solution. The insoluble product was filtered and washed with water until the washings were no longer alkaline. Upon two crystallizations from dilute propyl alcohol a tetra-acetyl derivative melting at 105–107° was obtained.

Anal.—Calcd. for $C_{28}H_{20}O_{12}$: C, 60.21; H, 5.41. Found: C, 60.34; H, 5.75.

Diethyl ester of dehydrodiprotocatechuic Acid

Two grams of dehydrodiprotocatechuic acid was suspended in 75 cc. of absolute ethyl alcohol, and the ester was prepared according to the procedure used in the preparation of the propyl ester. A yield of 1.1 Gm. or 46.5% of white crystalline product melting at 257–259° was obtained after three crystallizations from dilute ethyl alcohol. The sample for analysis had a melting point of 259.5–260.5°.

Anal.—Calcd. for $C_{18}H_{18}O_8$: C, 59.67; H, 5.01; EtO, 24.86. Found: C, 59.23; H, 5.10; EtO, 24.69, 24.67.

The tetra-acetate was prepared. Melting point, 144–146°.

Anal.—Calcd. for $C_{26}H_{26}O_{12}$: C, 58.86; H, 4.94. Found: C, 59.09; H, 5.18.

Dimethyl ester of Dehydrodiprotocatechuic Acid

The dimethyl ester of dehydrodiprotocatechuic acid was prepared according to the procedure used in the preparation of the propyl ester. From 2 Gm. of dehydrodiprotocatechuic acid, 1.6 Gm. (73.4% yield) of a product was isolated. This ester was crystallized from dilute methyl alcohol and it had a melting point of 286–288°.

Anal.—Calcd. for $C_{18}H_{14}O_4$: C, 57.49; H, 4.22; MeO, 18.56. Found: C, 57.38; H, 4.57; MeO, 18.68, 18.57.

The tetra-acetyl derivative was prepared as described above. Melting point, 134–136°.

Anal.—Calcd. for $C_{24}H_{22}O_{12}$: C, 57.37; H, 4.41. Found: C, 57.01; H, 4.61.

Dehydrodicreosol or 2,2′-Dihydroxy-3,3′-dimethoxy-5′,5′-dimethyl Biphenyl

A. By the Oxidative Coupling of Creosol.—Six cubic centimeters of creosol was dissolved in 1000 cc. of distilled water and the solution chilled to 5°. Ten cubic centimeters of ferric chloride solution (9 Gm. of $FeCl_3 \cdot 6H_2O$ in 100 cc. of water) was added slowly with constant shaking, and the mixture was allowed to stand at room temperature for one week. The precipitated product was collected by filtration which amounted to 0.7 Gm. of a rather crude sticky product. Two crystallizations from dilute alcohol produced 0.2 Gm. of a white crystalline dehydrodicreosol, melting at 130–132°.

B. By the Reduction of Dehydrodivanillin.—Ten grams of dehydrodivanillin, 125 cc. of concentrated hydrochloric acid, 125 cc. of 95% alcohol, 25 Gm. of amalgamated zinc dust, and 75 cc. of water were heated under the reflux for an hour. Then a second 25 Gm. of amalgamated zinc dust was added, and the mixture was refluxed for an hour after solution was effected. The time required for solution varied, but it was usually complete in two hours. The mixture was filtered while hot to remove excess amalgamated zinc. Upon cooling a precipitate was obtained, which was collected by filtration. An additional precipitate resulted upon the addition of water to the filtrate. About 9 Gm. of crude product having a melting range of 107–115° was obtained. This product was purified by fractional solution in dilute alcohol. The concentration of alcohol was held to a minimum so as to dissolve the least amount of oxidized reddish tarry material, which was removed by filtration of the hot solution. The process was repeated several times. After the major portion of the tarry material had been removed by this process, the product was recrystallized from a higher concentration of alcohol, producing 2.5 Gm. of light pink crystals which melted at 127–129°. The sample for analysis was repeatedly recrystallized from dilute alcohol until a white product was obtained. Melting point, 130–132°.

Anal.—Calcd. for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61; MeO, 22.62. Found: C, 70.05; H, 6.91; MeO, 22.47, 22.59.

The diacetyl derivative was prepared by the usual method. Melting point, 162–163°.

Anal.—Calcd. for $C_{20}H_{22}O_6$: C, 67.02; H, 6.19. Found: C, 66.67; H, 6.56.

The dibenzoate was prepared. Melting point, 193–195°.

Anal.—Calcd. for $C_{30}H_{26}O_6$: C, 74.67; H, 5.43. Found: C, 74.70; H, 5.78.

Nordehydrodicreosol or 2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl

Three grams of dehydrodicreosol was demethylated with the glacial acetic acid-hydrobromic acid mixture previously described to yield 0.7 Gm. of a light pink crystalline product. Melting point, 220–222°.

Anal.—Calcd. for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 68.21; H, 6.02.

The tetra-acetate was prepared as previously described. Melting point, 110–112°.

Anal.—Calcd. for $C_{22}H_{22}O_8$: C, 63.76; H, 5.35. Found: C, 63.58; H, 5.49.

Guaiacyl Valerate

Miller, Hartung, Rock, and Crossley (10) reported a general procedure for the preparation of guaiacol esters. This procedure was used to prepare guaiacyl valerate. Excess thionyl chloride was removed from the reaction mixture by distillation rather than by washing with a bicarbonate solution. The fraction distilling between 255–265° was collected and used for the next reaction. A yield of 80% of the ester was obtained.

4-*n*-Vateryl Guaiacol

4-*n*-Vateryl guaiacol was prepared according to the procedure of Coulthard and co-workers (11) with some modifications. No attempt was made to separate a portion of the aluminum chloride complex that had separated from the reaction mixture. To the reaction mixture 500 cc. of hydrochloric acid (1:1) was added slowly, and the mixture was warmed on the steam bath to dissolve all the precipitate. The nitrobenzene layer was separated and washed twice with 100-cc. portions of water. The nitrobenzene solution was then steam distilled to remove nitrobenzene, and the remaining oil was distilled under 15 mm. pressure. The fraction distilling between 190–200° was collected and placed in the refrigerator overnight. The solidified mass was then filtered on a Büchner funnel to remove a yellow oil which accompanied the product. Crystallization from 95% alcohol yielded 30.3 Gm. of 4-*n*-vateryl guaiacol that melted at 59–61° (reported 60–62°). An additional quantity of 6 Gm. was recovered from the mother liquor.

4-*n*-Amyl Guaiacol

The carbonyl group of 4-*n*-vateryl guaiacol was reduced by the Clemmensen method. Twenty grams of 4-*n*-vateryl guaiacol, 60 Gm. of amalgamated zinc dust, 120 cc. of hydrochloric acid (1:1), and 40 cc. of alcohol were heated under reflux for twelve hours. The reaction mixture was allowed to cool, and the excess amalgamated zinc dust was removed by filtration. The alcohol was distilled off on the steam bath, and the remaining liquid was extracted with 150 cc. of ether. The ether solution was then washed with 25 cc. of water and dried over anhydrous sodium sulfate. After distillation of the ether, there remained 14 Gm. of oil. This oil was distilled under 15 mm. of pressure, and the fraction distilling between 155–157° was collected. 4-*n*-amyl guaiacol thus obtained was colorless, but became light yellow upon standing.

Dehydrodi-(4-*n*-amyl Guaiacol) or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-di-*n*-amyl Biphenyl

Eight cubic centimeters of 4-*n*-amyl guaiacol was dissolved in 3000 cc. of 40% alcohol, and a solution of ferric chloride, prepared by dissolving 14 Gm. of $FeCl_3 \cdot 6H_2O$ in 100 cc. of 50% alcohol, was added through a dropping funnel with constant stirring. The addition required about three hours. The mixture was allowed to stand for twenty-four hours, and the product that precipitated was filtered. The precipitate was washed with 15 cc. of petroleum benzin (U. S. P.), which yielded 3.6 Gm. of light tan-colored product. After two crystallizations from

dilute alcohol 3 Gm. of dehydrodi-(4-*n*-amyl guaiacol) were obtained. Melting point, 114–116°.

Anal.—Calcd. for $C_{34}H_{54}O_4$: C, 74.58; H, 8.87; MeO, 16.07. Found: C, 74.36; H, 8.56; MeO, 16.03, 16.01.

The diacetate was prepared. Melting point, 70–73°.

Anal.—Calcd. for $C_{38}H_{58}O_6$: C, 71.46; H, 8.14. Found: C, 71.14; H, 8.00.

Nordehydrodi-(4-*n*-amyl Guaiacol) or 2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl

The demethylation of dehydrodi-(4-*n*-amyl guaiacol) was accomplished by the above-described method. From 3 Gm. of dehydrodi-(4-*n*-amyl guaiacol), 1.8 Gm. of nordehydrodi-(4-*n*-amyl guaiacol) melting at 146–148° was obtained.

Anal.—Calcd. for $C_{32}H_{50}O_4$: C, 73.71; H, 8.44. Found: C, 74.04; H 8.53.

The tetra-*p*-nitrobenzoate was prepared. Melting point, 152–154°.

Anal.—Calcd. for $C_{60}H_{62}O_{16}N_4$: C, 62.88; H, 4.43. Found: C, 63.00; H, 4.56.

Ferric Chloride Color Tests

Ferric chloride color tests were made on all substituted dehydrobiguaiacols and dehydrobicatechols which were prepared in this investigation. In most cases it was necessary to have a rather concentrated alcoholic solution of the compound before true color changes were noticeable. With ferric chloride test solution all the alcoholic solutions of the dehydrobicatechols gave a very transient green color, which immediately turned blue. On addition of sodium bicarbonate solution the color changed to a dark red. The alcoholic solution of dehydrobiguaiacols gave a green color with ferric chloride.

SUMMARY

The synthesis of six new 5,5′-disubstituted 2,2′,3,3′-tetrahydroxy biphenyls was carried out by the oxidative coupling of 4-substituted guaiacols and subsequent demethylation of the bis compounds or by some modifications thereof. In the alkyl substituted 2,2′,3,3′-tetrahydroxy biphenyls the methyl, *n*-propyl, and *n*-amyl compounds were prepared, and in the carboxylic acid substituted compounds (dehydrodiprotocatechuic acid) the methyl, ethyl, and *n*-propyl esters were prepared.

Antioxidant tests were made. The alkyl substituted dehydrobicatechols showed marked activity. On a weight-in-weight basis the activity decreased as the alkyl chain increased. The esters of dehydrodiprotocatechuic acid showed very little activity.

The dehydrobicatechols showed marked bacteriostatic activity when a synthetic chemical medium was used. The bacteriostatic activity was destroyed by 1 per cent blood serum.

REFERENCES

(1) Cousin, H., and Herissey, H., *Bull. soc. chim.* (*France*), **3**, 1066(1908); *Compt. rend.*, **146**, 1413(1908); Erdtman, H., *Biochem. Z.*, **258**, 177(1933).
(2) Tsuchiya, H. M., Drake, C. H., Halvorson, H. O., and Bieter, R. N., *J. Bact.*, **47**, 422(1944).
(3) Lundberg, W. O., Halvorson, H. O., and Burr, G. O., *Oil and Soap*, **21**, 33(1944).
(4) Gisvold, O., Buelow, D., and Carlson, E. H., THIS JOURNAL, **35**, 188(1946).
(5) Marsh, P. B., and Butler, M. L., *Ind. Eng. Chem.*, **38**, 701(1946).
(6) Brit. Patent 355,055 (Sept. 10, 1929); *Chem. Abs.*, **26**, 4198(1932).
(7) Elbs, K., and Lerch, H., *J. prakt. Chem.*, **93**, 1(1916).
(8) Erdtman, H., *Proc. Roy. Soc.* (*London*), **A143**, 191(1933).
(9) Tiemann, F., *Ber.*, **18**, 3493(1885).
(10) Miller, E., Hartung, W. H., Rock, H. J., and Crossley, F. S., *J. Am. Chem. Soc.*, **60**, 7(1938).
(11) Coulthard, C. E., Marshall, J., and Pyman, F., *J. Chem. Soc.*, **1930**, 280.

Notes

Note on the Determination of Estrone, Equilin, and Equilenin by Infrared Spectrophotometry*

By FREDERICK C. NACHOD, E. T. HINKEL, JR., and CALVIN ZIPPIN

THE SIMULTANEOUS DETERMINATION of the principal components of natural estrogenic materials by means of infrared spectra has been described recently by Carol, Molitor, and Haenni (1). They were able to show that the maxima at 10.45 μ, 10.88 μ, and 10.96 μ are characteristic for equilenin, estrone, and equilin, respectively, but at each wave length, the two other estrogens also contribute to the total observed absorption.

To allow for such contributions, the authors have suggested a method of successive approximations

* Received Sept. 13, 1948, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

which appears cumbersome and time consuming. A procedure is suggested herein which avoids these approximations by solving three simultaneous equations (2).

PROCEDURE

Since there are three discrete maxima for the three estrogenic compounds, one can write three simultaneous equations. C_a denotes the concentration of equilenin, C_b the concentration of estrone, and C_c the concentration of equilin; and k_a, k_b . . . , k_b' . . . , k_b'', k_c'' indicate the proportionality constants which are ratios of optical density divided by concentration. Thus is written:

$$O.\ D._{10\text{-}43} = k_aC_a + k_bC_b + k_cC_c \quad \text{(I)}$$
$$O.\ D._{10\text{-}88} = k_a'C_a + k_b'C_b + k_c'C_c \quad \text{(II)}$$
$$O.\ D._{10\text{-}96} = k_a''C_a + k_b''C_b + k_c''C_c \quad \text{(III)}$$

The equations (I), (II), and (III) can be solved for C_a, C_b, C_c after the k-values have been determined from suitable calibration charts. For example, taking data from the "working curves" of Fig. 3 of the paper by Carol, *et al.*, the nine finite k-values, e.g., the slopes of the curves can be inserted into these equations and the following is obtained:

$$O.\ D._{10\text{-}43} = \textit{0.0407}\ C_a + 0.0055\ C_b + 0.0097\ C_c \text{ (IV)}$$
$$O.\ D._{10\text{-}88} = 0.0066\ C_a + \textit{0.0531}\ C_b + 0.0165\ C_c \text{ (V)}$$
$$O.\ D._{10\text{-}96} = 0.0068\ C_a + 0.0216\ C_b + \textit{0.0374}\ C_c \text{ (VI)}$$

In the above, the major contributing terms, corresponding to the first approximation of Carol and collaborators, are italicized.

It is to be noted that all O. D. values are measurable and only three unknown quantities, C_a, C_b, and C_c, remain. By solving three simultaneous equations in the usual manner for C_a, C_b, and C_c, one obtains:

Concentration of equilenin:
$$C_a = 25.65\ O.\ D._{10\text{-}43} + 0.03\ O.\ D._{10\text{-}88} - 6.68\ O.\ D._{10\text{-}96} \quad \text{(VII)}$$

Concentration of estrone:
$$C_b = 22.95\ O.\ D._{10\text{-}88} - 2.12\ O.\ D._{10\text{-}43} - 9.57\ O.\ D._{10\text{-}96} \quad \text{(VIII)}$$

Concentration of equilin:
$$C_c = 33.48\ O.\ D._{10\text{-}96} - 3.44\ O.\ D._{10\text{-}43} - 13.26\ O.\ D._{10\text{-}88} \quad \text{(IX)}$$

In a hypothetical example where C_a was assumed to be 6 mg./ml., C_b 4 mg./ml., and C_c 8 mg./ml., the expected optical densities at the three wave lengths were computed by adding the individual contributions of the three estrogens. Substituting these values into equations (VII), (VIII), and (IX), the calculated C-values were: C_a = 6.10 mg./ml., C_b = 3.95 mg./ml., and C_c = 7.94 mg./ml.

The k-values here reported are based on the experimental data of Carol and co-workers. Due to differences between instruments as well as possibly between calibration compounds, the numerical values may have to be revised slightly by other workers in the field, to fit their specific needs. However, the principle here employed will be applicable in all cases.

SUMMARY

The method of Carol and co-workers for the determination of estrogenic materials by successive approximations has been extended and subjected to mathematical treatment. By solving three simultaneous equations and by using numerical values for proportionality factors, the calculation of estrogenic materials can be simplified.

REFERENCES

(1) Carol, J., Molitor, J. C., and Haenni, E. O., THIS JOURNAL, 37, 173(1948).
(2) Cf. Stewart, D. W., in Weissberger, A., "Physical Methods of Organic Chemistry," Interscience Publishers, New York, Vol. 2, 1946, p. 1300.

Vitamins B_1. A Note on the Stability of Thiamine Hydrochloride in Aqueous Solution in the Presence of Iodine and Potassium Iodide*

By HUBERT W. MURPHY and JOHN M. GOODYEAR

DURING the investigation of the formation of a precipitate which was produced when Lugol's Solution was added to an elixir of thiamine hydrochloride made with sucrose and the absence of such a precipitate when the elixir was made with glucose, the stability of thiamine hydrochloride in such mixtures was studied simultaneously. The immediate formation of a precipitate apparently is prevented in the elixir made with glucose by absorption of the iodine on dextrins.

Since inquiry has been made with regard to the stability of thiamine hydrochloride in the presence of iodine and iodides and the opinion expressed (1) is at variance with our data, it would appear desirable to correct this concept.

EXPERIMENTAL

Typical data reproduced in Table I indicate that, within experimental error, the presence of iodine and

* Received July 17, 1948, from the Chemical Control Division, Eli Lilly and Company, Indianapolis 6, Ind.

iodides does not exert a destructive effect upon thiamine hydrochloride in aqueous solutions. This stability was studied within a *p*H range of 2.0 to 4.8. All samples were stored in amber glass bottles in diffused sunlight at room temperature which varied from 23° to 33°. The presence of ethyl alcohol in concentrations up to 15% had little or no effect upon the stability of the thiamine in these preparations.

TABLE I.—STABILITY OF THIAMINE HYDROCHLORIDE IN AQUEOUS PREPARATIONS CONTAINING IODINE AND POTASSIUM IODIDE

Preparation	Thiamine Hydrochloride Found Expressed as % of That Originally Present			
	1 Year	2 Years	3 Years	4 Years
A	..	86	82	85
B	..	88	88	86.5
C	92	88	87	86

PREPARATION A

Glucose	37.5 Gm.
Thiamine Hydrochloride	0.348 Gm.
Lugol's Solution	2.0 cc.
Water, Distilled, *q. s.* to make	100 cc.

PREPARATION B

Sucrose	25.0 Gm.
Thamine Hydrochloride	0.376 Gm.
Lugol's Solution	2.0 cc.
Water, Distilled, *q. s.* to make	100 cc.

PREPARATION C

Glucose	37.5 Gm
Thiamine Hydrochloride	0.40 Gm.
Hydrochloric Acid U.S.P. (*q. s.* to adjust *p*H to 3.0)	
Water, Dist., *q. s.* to make	100 cc.

The *p*H of each of the first two mixtures was approximately 2.20 and C was approximately 3.0 throughout the four-year period of observation. Assays were made by the standard U. S. P. thiochrome method. Blanks were normal and displayed no extraneous fluorescence. The assays recorded for the end of the four-year aging period were made by both the thiochrome method and the microbiological yeast fermentation method. Very close agreement in the results of both methods was observed.

CONCLUSIONS

The presence of glucose in an elixir of thiamine hydrochloride prevents the immediate formation of a precipitate upon the addition of 2% by volume of Lugol's Solution.

The stability of thiamine hydrochloride in aqueous solution within the *p*H range of 2.0 to 4.8 does not appear to be influenced by the presence of glucose as a replacement for sucrose or by ethyl alcohol in a concentration up to 15%. The presence of iodine and potassium iodide in concentrations equivalent to 2% of Lugol's Solution by volume does not exert a destructive effect on thiamine hydrochloride in aqueous solutions in the presence of glucose or sucrose with or without the addition of up to 15% alcohol within the *p*H range of 2.0 to 4.8. There should be no objections to combinations of iodine or iodides and thiamine hydrochloride in mixtures of this type.

REFERENCE

(1) THIS JOURNAL, *Pract. Pharm. Ed.*, **8**, 379(1947).

Book Reviews

New and Nonofficial Remedies, 1948. Issued under the direction and supervision of the Council on Pharmacy and Chemistry of the American Medical Association. J. B. Lippincott Company, Philadelphia, 1948. li + 800 pp. 12 x 19 cm. Price $3.00.

New and Nonofficial Remedies is one of the most valuable books in the pharmacist's library. Although it is written for physicians, pharmacists have adopted it and follow the appearance of new editions with much interest.

As each year's issue of this handy book became available we noticed it was thicker than the previous one. The 1948 issue has become so large that the publishers may find it necessary to change the format somewhat. Because we appreciate the problem that the increase in size has brought and because we recognize some of the dilemmas facing the Council on Pharmacy and Chemistry, these few suggestions are ventured in the hope that they will help in planning future editions.

First there is the matter of scope. The real value, it seems to us, in this excellent work lies in the *newness* and the *nonofficial* character of the drugs.

The Council very wisely has a rule to eliminate drugs which have been official for over twenty years, although we are inclined to wonder if the time should not be shortened now that the U. S. P. and the N. F. are revised every five years. Nevertheless in view of this rule, one is surprised to find agar, barbital, barium sulfate, carbromal, ephedrine, petrolatum, phenobarbital, quinidine sulfate, quinine sulfate, silver nitrate, theophylline, thymol iodide, and others still present. Many of the nonofficial drugs which are over twenty years old are still recognized, too—witness Afenil, bismuth tribromophenate, metacresyl acetate, Digalen, ethyl salicylate, ferrous lactate, Orthoform, scarlet red sulfonate, sodium peroxide, and many other drugs.

In making a hasty count, it was noted that nearly half of the drugs in the 1948 edition of N. N. R. are at present official in either the U. S. P. or the N. F. In the category of local anti-infective drugs, 23 are nonofficial, 19 in the N. F. and 9 in the U. S. P. In the systemic anti-infective group, 11 are nonofficial, 4 are N. F. and 18 are U. S. P. Among the autonomic drugs, 18 are nonofficial and 10 are in the U. S. P. Eighteen hormones and synthetic

substitutes are nonofficial and 9 are in the U. S. P.; 11 vitamin drugs are nonofficial and 15 are in the U. S. P., etc.

Perhaps one of the most difficult achievements of any work pertaining to drug standards is consistency. N. N. R. 1948 is full of inconsistencies, many of which can be easily corrected. For example, the Council desires to foster generic names and yet often gives emphasis to trade-marked names apparently unintentionally. Generic names are in bold face capitals to distinguish them from protected names which are in bold face upper and lower case, i.e., AMOBARBITAL—Amytal—Lilly. However, in the case of substances for which generic names have not yet been adopted, the registered trade-mark name appears in bold face capitals, i.e., DIGIFOLIN—Ciba. This seems to give a slight advantage to those firms which have not complied with the Council's request.

In the preservative field, the generic versus protected names issue seems to be particularly unfairly treated at times. For example, on page 489 under Influenza Virus Vaccine it is noted that Lilly's product is preserved with "Merthiolate" while for the Squibb, Sharp and Dohme, and the Pitman-Moore products the chemical name "sodium ethyl mercuri thiosalicylate" is employed.

In a number of places it is evident that a standard style book would be of great assistance. The basic arrangement in subsections seems to be alphabetical. However, on pages 52–53, Butethamine Formate and Hydrochloride appear between Metycaine Hydrochloride and Phenacaine Hydrochloride evidently because they are alphabetized under their trade names, Monocaine Formate and Hydrochloride.

The treatment of "salts" of organic compounds is not always uniform. In the case of the sulfonamides, the sodium compounds are all placed together in a separate section following the sulfonamides themselves (pp. 144–148). In the case of the soluble barbiturates, the sodium compound follows the "acid" form, i.e., amobarbital is followed by amobarbital sodium (see pp. 448 ff.).

Dosage forms of a drug are not all treated uniformly either. For example, epinephrine, epinephrine solution, and epinephrine in oil each rates a separate monograph (pp. 233–241). In the case of penicillin, there is one basic monograph which covers all recognized penicillin dosage forms.

Frequently there is an inconsistency in the chemical nomenclature (especially noticeable in the barbiturates), which is most confusing to physicians, pharmacists, and even to organic chemists. A similar inconsistency is the alphabetizing of isomers, i.e., *meta*cresyl acetate is under *c* but *para-aminobenzoic acid* is under *p*.

A valuable change in N. N. R., actually brought about in 1947, is the separation of the tests and standards from the "physicians' part of the book." In general, Section B on tests and standards is well done and is useful. Correlating tests a little more closely with U. S. P.-N. F procedure would be very helpful to the research workers on these compendia since frequently new admissions to these official books come from N. N. R.

Because of the rapid appearance of "miracle" drugs, each year sees the book becoming a little more unwieldy. It is believed that a careful examination of scope and the development of a consistent style can obviate many of these difficulties. It is in this spirit that the reviewer has the temerity to call attention to these matters.

The editors, the members of the Council, the members of the laboratory staff, and all who participated in this enterprise are to be congratulated upon doing an amazing volume of work in a very short time and making a real contribution to the pharmaceutical and medical professions.

Trade-Mark Law and Practice. Lanham Act Edition. By LEON H. AMDUR. Clark Boardman Company, New York, 1948. xiii + 776 pages. 15 x 24 cm. Price $15.00.

For many years execution of the trade-mark law, like patent law, has been a specialized and an essential operation. Since a large number of drugs and drug products have trade-marked names, this phase of law is of tremendous importance to the drug industry.

This book gives the Trade-Mark Act of 1946 (the Lanham Act), the Trade-Mark Rules of the U. S. Patent Office and an interpretation of the many factors in trade-mark law by citing cases.

Descriptiveness, geographical connotations, surnames and corporate names, secondary meaning, radio-marks, generic designation, similarity of goods, related companies, abandonment and many other problems relating to the trade-mark are as important to the drug field as to other fields. Nearly any problem in trade-mark law in this book cites a case in the drug field. The book is well documented with case citations and is indexed by means of a general index, an index to patent office forms, an index to the sections of the 1946 Act referred to in text, an index to prior acts, and an index to patent and trade-mark rules referred to in text.

To anyone interested in the intricacies of trade-mark law, Mr. Amdur's book will be of material assistance.

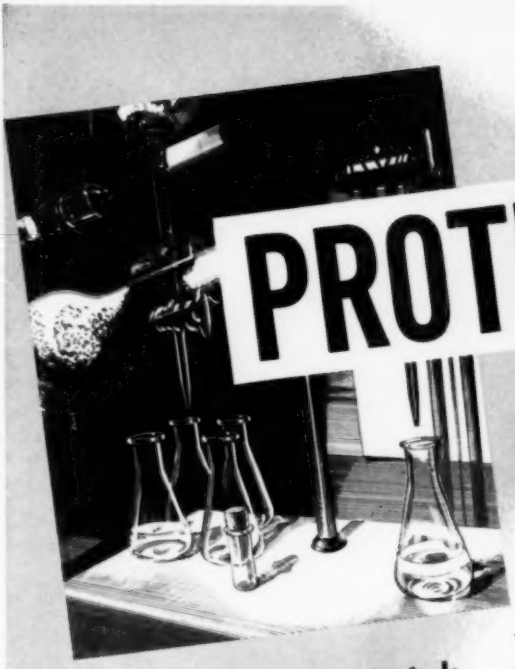
Practice of Allergy. 2nd ed. By WARREN T. VAUGHAN and J. HARVEY BLACK. C. V. Mosby Company, St. Louis, 1948. xx + 1132 pp. 16 x 25 cm.

Although *Practice of Allergy* was written for the medical practitioner, some pharmacists will have occasion to consult it for reference.

The history of allergy and anaphylaxis makes fascinating reading as do the more general phases of allergy. Climate, social status and environmental factors in relation to the incidence of allergy are of general interest to all in the health field. By sharpening the pharmacists' interest in allergy, no doubt many seekers after advice on self-medication will be sent to proper physicians.

Some pharmacists, particularly those practicing in hospitals, will be interested in the detailed methods for preparing and standardizing allergens for diagnostic testing and for desensitization. Others will find interest in techniques of pollen counting and identification of pollens and other allergens (aerobiology). Of especial importance is the section of the book dealing with drug allergy.

A short chapter on treatment using antihistaminics and other drugs is also included.



PROTECTION

for your ANTACID PREPARATIONS

Which of these Baker Fine Chemicals best suits your formula?

ALUMINUM HYDROXIDE
Available in three densities

MAGNESIUM OXIDE
Three different types and densities

BISMUTH SUBGALLATE
A clear yellow powder

BISMUTH SUBSALICYLATE
High Chemical Purity

BISMUTH SUBCARBONATE
Four densities and a suspension grade

BISMUTH SUBNITRATE
High purity for ingredient use or synthesis

MAGNESIUM CARBONATE
Two uniform densities

MAGNESIUM HYDROXIDE
For making tablets

MAGNESIUM TRISILICATE
Medium and light densities

MAGNESIUM PHOSPHATE
A free-flowing powder

Your company is proud of the formula it has developed for an antacid preparation.

Through rigid manufacturing and continued laboratory control you intend to maintain the reputation of that product and your good name.

Nothing is more essential to the fulfillment of such an intention than insistence *at all times* on quality fine chemicals—the vital ingredients of any antacid formula.

It is important that each fine chemical be the *right* one for your formula—that each be extremely pure—that each be regularly available to uniform physical standards. These things are your protection.

On this page are listed several Baker Fine Chemicals for antacid preparations. In this list, or in the Baker line, there is the *right* one for you. Whichever it is, that fine chemical brings you purity *to the decimal*, and is available on a tonnage basis.

No matter what product you manufacture—if it requires one or more fine chemicals of *measured purity*—specify Baker!

Address: *Fine Chemical Division*, J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Baker's Chemicals

C. P. ANALYZED • FINE • INDUSTRIAL

IDEAL ADSORBENTS

FOR THE BIOLOGICAL INDUSTRY BY Filtrol

FILTROL ADSORBENTS

Filtrol activated adsorbents are gaining wide popularity in the manufacture and estimation of anti-biotics, vitamins, and other biological products. These adsorbents are pure, highly-refined powders prepared from selected Bentonite clays by perfected Filtrol methods of chemical activation. Filtrol adsorbents possess selective adsorptive capacity for a number of the vitamins and anti-biotics and they are carefully standardized to perfect uniformity during manufacture.

FILTROL DESICCANTS*

Special small size packets of Desiccite #25 are now being widely supplied to pharmaceutical houses for individually bottled preparations such as vitamins and plasmas.

PROPERTIES:

	SUPER FILTROL*	FILTROL* X-202	FILTROL* THIAMIN GRADE
USE:	GENERAL ADSORBENT FOR ANTI-BIOTICS AND VITAMINS	CHROMATOGRAPHIC ADSORBENT FOR VITAMINS	GENERAL ADSORBENT FOR THIAMIN
PHYSICAL STATE:	Powder	Powder	Powder
COLOR:	Light Grey	White	Light Grey
ODOR:	Odorless	Odorless	Odorless
KEEPING QUALITIES:	Stable	Stable	Stable
WEIGHT PER CU. FT. (LBS.)	45	45	45
SURFACE AREA (SQ. M. PER GR.**)	300	300	300
FREE MOISTURE	13.0-16.0 %	13.0-16.0%	13.0-16.0%
pH (approx.)***	3	3	3
MESH: (T-200) %	93-95	84-98	93-95

** Approx. after 650° F Calcination as determined by Nitrogen.
***Of SLURRY

Filtrol®

WORLD'S LARGEST MANUFACTURERS OF ACTIVATED ADSORBENTS

GENERAL OFFICES:
634 South Spring St., Los Angeles 14
PLANTS:
Vernon, California
Jackson, Mississippi

write for name of agent in your country
ecrire pour le nom de l'agent du votre pays
escribir pidiendo el nombre del agente en su pas

*T. M. Reg. U. S. Pat. Off.

SEND COUPON TODAY

FILTROL CORPORATION

GENERAL OFFICES

634 SOUTH SPRING STREET, LOS ANGELES 14, CALIFORNIA

GENTLEMEN:

We are interested in securing more information about Filtrol adsorbents:

☐ Super Filtrol ☐ Filtrol X-202 ☐ Filtrol Thiamin Grade

Please send us a sample of:

☐ Super Filtrol ☐ Filtrol X-202 ☐ Filtrol Thiamin Grade

FIRM NAME_____

ATTENTION_____

STREET_____

CITY_____ ZONE_____ STATE_____

☐ We are interested in securing more information about Desiccite #25 . . . the profitable method of eliminating losses of potency and quality due to moisture.